

HEAT-STABLE EXTRACELLULAR PROTEASES OF
PSYCHROTROPHIC PSEUDOMONADS:
PURIFICATION AND PHYSICOCHEMICAL
PROPERTIES

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FRANCIS M. BARTLETT



HEAT-STABLE EXTRACELLULAR PROTEASES OF
PSYCHROTROPHIC PSEUDOMONADS:
PURIFICATION AND PHYSICOCHEMICAL PROPERTIES

BY

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A thesis submitted
in partial fulfillment
of the requirements for the
degree of Doctor of Philosophy

Department of Biology

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ABSTRACT

There is a trend in the dairy industry towards the storage of bulk raw milk at refrigerated temperatures for extended periods of time before processing. This practice has the disadvantage of providing conditions favourable for the growth of psychrotrophic (cold-tolerant) microorganisms. Most raw milk psychrotrophs are heat sensitive Gram-negative bacteria, many of which produce heat-resistant extracellular proteases. The heat-stability of these enzymes enables them to retain some activity in milk following conventional pasteurization, as well as ultra-high temperature (UHT) pasteurization. The heat-resistant proteases have been implicated in such defects as the development of off-flavours, gelation and clearing of milk, and reductions in cheese yields.

The heat-stable extracellular proteases of six psychrotrophic pseudomonads isolated from raw milk were purified to homogeneity by affinity chromatography, using CBZ-DL-phenylalanine TETA Sepharose-4B. The yields of purified enzymes were 38 to 59% before concentration by ultrafiltration. The molecular weights, which were determined by gel-filtration, were 38 to 40kD, and the proteins had isoelectric points (pI) from 5.7 to 6.2. The optimum pH for proteolytic activity was between 7 and 8, while the optimum temperature ranged from 30 to 40°C. A dramatic loss of activity was noted for each protease at 45°C.

Each of the proteases were metalloproteases as indicated by their sensitivity to the metal chelating agent EDTA. Restoration of activity to EDTA-treated proteases was achieved by the addition of Mg, Mn or Ca ions. The predominant

metal ion present in the three proteases examined was Ca (5.8 g-atoms/mole) followed by Mg and Zn, with trace amounts of Mn. The amino acid content of each of the proteases were similar, with high numbers of aspartic acid, serine, glycine, and alanine residues. The N-terminal amino acid determined for three of the proteases was found to be threonine. The T16 protease contained two aminosugars, glucosamine and galactosamine, and was classified as a glycoprotein.

Five of the six proteases exhibited a preference for α -casein as a protein substrate, while T16 showed greatest activity towards κ -casein. None of the proteases was able to break down the whey proteins, α -lactalbumin, or β -lactoglobulin. Collagenase and elastase activities were noted for each protease. The T16 protease was quite stable, with a D-value at 150°C of 2.2 min. The presence of Ca or Mg ions stabilized the T16 protease against inactivation at 90°C for 10 min while lactose had no effect. The secondary structure of the T16 protease consisted of "random coil" (67%) and β -structure (33%) with no α -helix. Upon heating from 45 to 55°C the secondary structure tended to become more random.

All of the proteases cross-reacted with the anti-serum to the T16 protease, including the protease of psychrotrophic pseudomonads from Ontario, British Columbia, Ireland, U.S.A., Australia and the Netherlands. An enzyme-linked immunosorbent assay (ELISA) was developed using anti-T16 IgG, which was sensitive to a minimum concentration of 720 μ g/ml of purified T16 protease. The mole % G+C content of the DNA psychrotrophic pseudomonads from different geographical regions were similar (58 to 82%) with the exception of 015 (44%).

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List of Abbreviations

BAPA	α -N-benzoyl-DL-arginine-p-nitroanilide
BSA	bovine serum albumin
CBZ or Z	benzyloxycarbonyl
CD	circular dichroism
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DNS-Cl	dansyl chloride
E _a	activation energy
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunoabsorbent assay
FFA	free fatty acid
G-6-P	glucose-6-phosphate
G + C	guanine and cytosine
Hb	hemoglobin
HIC	hydrophobic interaction chromatography
HRPO	horse radish peroxidase
HTST	high temperature short time
IgG	immunoglobulin
LPL	lipoprotein lipase
LTI	low temperature inactivation
MFGM	milk fat globule membrane
NADP	β -nicotinamide adenine dinucleotide phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PCMB

p-chloromercuribenzoate

PMSE

phenylmethylsulfonyl fluoride

SDS

sodium dodecyl sulphate

TAME

p-toluenesulphonyl-L-arginine methyl ester

TEMED

N,N,N,N'-tetramethylethylene diamine

TCA

trichloroacetic acid

TES

tris[hydroxymethyl] methyl-2-aminoethanesulfonic acid

Tris

hydroxymethylamino methane

Tm

melting temperature

TSA

tryptic soy agar

TSB

tryptic soy broth

UHT

ultra high temperature

Dedicated to my parents.

Chapter 1

INTRODUCTION

1.1. General

Liquid milk is a highly perishable food, which has considerable nutritional and economical importance. The nutritive value of milk is well documented (Jakobsen, 1978). Milk alone provides all of the nutrients required by the newborn. In developed countries, 20-30% of the population's dietary protein is provided by milk and milk products. According to Statistics Canada (1986) the production of bovine milk in Canada for 1985 was in excess of 7 million kiloliters, with a cash value of approximately 3 billion dollars. Much of this milk was purchased by the dairy industries, which processed it into a variety of products such as 2% milk, evaporated milk, powdered milk, cheese, buttermilk, yogurt, and ice cream.

The major problem for the dairy industry is the poor keeping quality of milk. This characteristic of milk is not surprising because milk was intended to be passed immediately from the mother to the newborn. Historically, the rapid spoilage of milk was the impetus for the development of various fermented dairy products such as cheese, yogurt, and buttermilk. These products are less susceptible to spoilage than liquid milk and as a result can be stored for longer periods of time (Rose, 1982).

There are a number of factors which contribute to the deterioration of milk. Some are inherent to the milk, and include lipid oxidation and the activities of endogenous enzymes. Others are external factors resulting from the growth of microorganisms (Allen and Joseph, 1985).

1.2. Inherent factors causing milk spoilage

1.2.1. Lipid oxidation in milk

The major chemical reactions which result in milk spoilage are those involved in lipid oxidation. This is the oxidation of unsaturated fatty acid residues in milk to form intermediate peroxides which decompose to yield rancid off-flavoured compounds such as ketones, aldehydes, alcohols, hydrocarbons, acids, and epoxides (Richardson and Korycka-Dahl, 1984). A variety of catalysts or pro-oxidants, such as metal ions, ascorbic acid, carotenoids, riboflavin and light, have been found to favour lipid oxidation in milk (Allen and Joseph, 1985).

1.2.2. Endogenous enzymes

The biochemical changes in milk are primarily a result of proteolytic and lipolytic enzymes. These enzymes may be of endogenous origin i.e. produced by the animal, or they may be produced by microbial contaminants.

Proteolytic enzymes

The presence of naturally occurring proteases in milk was first reported in 1897 (Eigel *et al.*, 1979). This endogenous milk protease is believed to be plasmin, a serine protease, which probably originates from the bovine plasma and

subsequently is transported across the mammary epithelial cells (Humbert and Alais, 1979). The native protease and plasmin are similar with respect to pH optimum, heat stability, and sensitivity to various inhibitors, as well as exhibiting antigenic relatedness (Eigel *et al.*, 1979).

The concentrations of plasmin and plasminogen in milk are low, 0.14-0.73 $\mu\text{g/ml}$ and 0.55-2.75 $\mu\text{g/ml}$, respectively, and appear to be dependent on the stage of lactation and health of the cow (Richardson, 1983). The enzyme is thought to be associated with the casein micelle in milk.

Like trypsin, plasmin is highly specific for bonds adjacent to lysine residues, and in milk is most active on β -casein to form γ -caseins (Humbert and Alais, 1979; Andrews, 1983). The αs_2 -casein is also very susceptible, while αs_1 -casein is hydrolyzed slowly. Kappa-casein and the whey proteins have been found to be very resistant to proteolysis (Snoeren *et al.*, 1979). Plasmin is quite heat stable and survives ultra high temperature treatments, 140°C for 4 s (Alichandrais *et al.*, 1980). It has been suggested that this native milk protease may contribute to the spoilage of UHT milk, however there has been very little evidence to support this view (Visser, 1981).

In addition to plasmin, there have been reports that thrombin, an acid milk protease, and aminopeptidases are also present in milk. However, their identity and relative concentrations have not been clearly established (Fox, 1981).

Lipolytic Enzymes

Lipases are a group of enzymes which hydrolyze lipids to free fatty acids resulting in the development of off-flavours in milk (Jensen, 1964). The only native lipolytic enzyme known in bovine milk is lipoprotein lipase (LPL). It is believed to result from leakage from the mammary gland into milk (Jensen and Pitas, 1976).

LPL is active against milk fat in natural globules only after the disruption of the milk fat globule membrane (MFGM). This may occur during agitation and homogenization of milk (Deeth and Fitz-Gerald, 1983). This enzyme has a molecular weight of 82,000, an optimum pH of 8.0 and is considered relatively unstable. It is inactivated by ultraviolet light, heat and oxidizing agents. LPL is primarily associated with the casein micelles, and it has been shown that caseins stabilize the enzyme against heat inactivation. However, HTST (72°C for 15 s) pasteurization is capable of almost complete inactivation of the enzyme.

1.3. Milk spoilage by microorganisms

Milk is an excellent medium for the growth of many microorganisms. Although the raw milk of healthy animals is essentially sterile when drawn, it readily becomes contaminated. The major sources of microorganisms in milk are the udder of the cow and the milking equipment, while water supplies, aerial contamination, and the milkers themselves may also contribute to the microbial load. The main groups of microorganisms which comprise the microflora of raw milk are micrococci, streptococci, asporogenous Gram-positive rods, Gram-negative rods and *Bacillus sp.* (Cousins and Bramley, 1981).

In the past, quality deterioration of milk was a result of inadequate cooling. This resulted in high acid development and off-flavours associated with lactic streptococci and other mesophilic bacteria (Elliot *et al.*, 1974). The common practice today of maintaining raw milk at refrigeration temperatures is quite effective in limiting the growth of lactic acid bacteria and the spoilage they cause (Law, 1979). Technological advances in the dairy industry have led to the trend towards fewer, yet larger, and more centralized dairy plants which can only operate efficiently if they have an adequate supply of milk at the start of processing. Consequently, raw milk tends to be maintained at refrigeration temperatures for extended periods of time. This would include storage in bulk tanks on the farm, transport to the dairy plant by refrigerated tanker trucks, and the time in holding tanks before processing. In all, raw milk may be held at low temperatures for 4 or more days (Stepaniak *et al.*, 1982). This has resulted in a dramatic shift in the dominant type of microorganisms in milk, from mesophilic lactic acid bacteria to psychrotrophic microorganisms (Richter, 1981).

1.3.1. Psychrotrophs and milk spoilage

The term psychrotroph was first proposed by Eddy (1960), and has been used by the dairy industry to describe those microorganisms able to grow at 7°C or less regardless of their optimum growth temperatures (Thomas and Thomas, 1978). Psychrotrophs, which include bacteria, yeasts and molds, are ubiquitous throughout the environment and are common contaminants of milk, as well as other foods including fish and meats (Kraft and Rey, 1979; Jay and Shalef, 1976; Greer, 1981; and Venugopal *et al.*, 1983). In milk produced under sanitary

conditions, psychrotrophs usually comprise less than 10% of the total microflora. However, this value can be in excess of 75% under unsanitary conditions (Cousins and Bramley, 1981). A study by Randolph *et al.* (1973) found that psychrotrophic bacteria accounted for more than 30% of the total bacteria in individual raw milk samples in Texas. Generally, within two days of storage at refrigeration temperatures, the microflora of raw milk is dominated by psychrotrophs (Driessen, 1981). Thomas (1974) studied the psychrotrophic bacteria of daily collected refrigerated bulk milk supplies, from bulk tanks and from road transport tankers on arrival at the dairy. He found that the counts of psychrotrophs had increased significantly by the time the milk arrived at the creamery. The rate at which the psychrotrophs increased in milk samples from a farm collection tanker was investigated and revealed that after two days of storage at 7°C the counts of psychrotrophs exceeded 10^6 colony forming units (cfu)/ml a level at which spoilage may be expected to occur (Cousins *et al.*, 1977).

The genera of psychrotrophic microorganisms commonly isolated from fresh raw milk include *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Enterobacter*, *Alcaligenes* and *Arthrobacter* (Cousin, 1982). The types of psychrotrophs may vary, however most have been characterized as heat-sensitive, Gram-negative, rods. Members of the genus *Pseudomonas* are the most frequent type isolated from raw milk, with *Pseudomonas fluorescens* as the most common representative (Law, 1979; Cousin, 1982). This, however, may be a result of the ease with which this species can be identified rather than a true reflection of its distribution (Law, 1979).

Significance of psychrotrophs

Psychrotrophs growing in raw milk present three problems to the dairy industry: (1) they can directly cause deterioration in large numbers (2) some psychrotrophs are heat-resistant and can survive pasteurization and (3) some psychrotrophs produce heat-resistant enzymes which retain activity even following exposure to pasteurization temperatures.

Direct spoilage by psychrotrophs

The presence of actively growing psychrotrophs in milk can cause spoilage by biochemically altering the constituents of milk. Some of the biochemical activities exhibited include the fermentation of carbohydrates with the liberation of acid and gas, the decomposition of urea, reduction of nitrate, production of pigments and the hydrolysis of starch, proteins, and lipids (Kraft and Rey, 1979; Richter, 1981). Such reactions can occur at low temperatures and the defects they cause may be detectable depending upon the size of the psychrotrophic population and the time of storage. Unsanitary practices before heat-treatment of raw milk can contribute a sufficiently large number of psychrotrophic microorganisms to cause spoilage (Busse, 1981). Post pasteurization contamination can also introduce psychrotrophic spoilage organisms. Commercially pasteurized milk generally becomes recontaminated with microorganisms, especially from non-sterile plant surfaces (Schroder, 1984). The spoilage of refrigerated pasteurized milk is usually a result of the growth of Gram-negative psychrotrophic recontaminants (Thomas and Druce, 1969; Moseley, 1980; and Schroder, 1984).

Schroder *et al.* (1982) were able to show that the shelf-life of pasteurized milk maintained at 5°C could be extended by several weeks in the absence of psychrotrophs. Similarly, Hankin *et al.* (1977) found a significant relationship between increased counts of psychrotrophs and decreased number of days at which pasteurized milk was judged to be bad.

Heat-resistant psychrotrophs

Microorganisms which survive HTST (72°C for 15 sec) and UHT (149°C for 4 s) pasteurization treatments of milk are not uncommon (Collins, 1981; Richter, 1981). Thermophilic psychrotrophic bacteria were first isolated from milk by Grosskroft and Harper in 1969. A study by Chung and Cannon (1971) found psychrotrophic sporeforming bacteria in 83% of the raw milk samples they tested. Psychrotrophic species of *Bacillus* were found in 30% of the 97 heat-treated milk samples which were examined by Shehata and Collins (1971).

Most of the thermophilic bacteria isolated from milk are sporeformers, which belong to the genus *Bacillus* (McKinnon and Pettipher, 1983). Nonsporeforming genera which may also be present in milk are *Microbacterium*, *Arthrobacter*, *Streptococcus* and *Corynebacterium* (Washam *et al.*, 1977; McKinnon and Pettipher, 1983; Batish *et al.*, 1985).

Even though heat-resistant sporeforming bacteria are in most milk supplies, their numbers are usually low. Mikolajcik and Simon (1978) found that of 109 milk samples 87% had psychrotrophic spore counts of less than 10/ml. Following

14 and 28 days of storage at 7°C, 50 and 83% of the samples had spore counts from psychrotrophs in excess of 100,000/ml respectively. Some of the defects in milk caused by these bacteria include bitter, fruity, rancid, and yeasty flavours, with sweet curdling being the most common type of spoilage (Collins, 1981). The sweet curdling defect has been attributed to *Bacillus cereus*.

However, due to the relatively low numbers of spores which are present in milk, in addition to a long lag period and slow growth at 7°C, spoilage by *Bacillus* species is not considered a practical problem until after two weeks of refrigerated storage (Thomas, 1974). In addition, Langeveld and Cuperus (1980) found that, at storage temperatures below 10°C, the likelihood of any heat-resistant contaminants outgrowing the heat labile Gram-negative rods, which recontaminate heat processed milk, is small. The sporeformers have a greater potential to cause spoilage in UHT-treated milk, which is free of recontaminants and maintained at room temperature for several months.

1.3.2. Heat-resistant microbial enzymes, lipases and proteases

Most raw milk psychrotrophs have been characterized as heat-sensitive, Gram-negative rods (Law, 1979; Cousin, 1982). These bacteria are readily destroyed by conventional pasteurization treatments and, as a result, are not present in heat-treated milk or milk products (except as post-pasteurization contaminants). In general the heat sensitive psychrotrophs themselves do not cause spoilage, however, many produce heat-stable extracellular enzymes which are capable of degrading important milk constituents (Law, 1979). The

degradative processes of lipolysis and proteolysis by the lipases and proteases of psychrotrophs are of concern to the dairy industry, particularly with respect to the keeping quality of pasteurized milk products.

Heat-stable lipases

The ability of Gram-negative psychrotrophs to produce heat-stable extracellular lipases has been demonstrated by a number of researchers (Driessen and Stadhouders, 1974; Law *et al.* 1978; Andersson *et al.* 1979; Adams and Brawley, 1981b). The lipolytic activity of such enzymes can lead to the development of rancidity in milk or the accumulation of short-chain fatty acids and partial glycerides which, in sufficient amounts, can be detected organoleptically (Jensen, 1964).

Chapman *et al.* (1976) found that the proportion of psychrotrophs which can produce lipolytic enzymes in raw milk can vary from less than 1% to more than 30%. Kishonti (1975) reported that 40% of the 60 strains of psychrotrophic bacteria isolated from bulk cooled raw milk, which included *Pseudomonas* spp., *Alcaligenes* spp., and *Enterobacter* spp., produced extracellular heat-resistant lipases. *Pseudomonas* species usually constitute the largest percentage of lipolytic psychrotrophs in raw milk (Law, 1979; Deeth and Fitz-Gerald, 1983; and Cousin, 1982).

Purification of a few lipases from *Pseudomonas* spp. has been reported, however, most studies of the lipases properties have been carried out on crude

enzyme preparations (Fox and Stepaniak, 1983). The pH optima for the activity of these lipases are in the range of 6.5-9.0, and they are stable over a wide pH range depending on the type of medium. The temperature optima have been reported to be from 25 to 70°C, while most fall between 30 to 45°C (Fox and Stepaniak, 1983). Many of these lipases actively hydrolyze lipids at the low temperatures used to store milk and milk products. Some lipases have even been found to be active below 0°C (Andersson, 1980).

Heat stability is an important property of lipases from psychrotrophs. Many of these enzymes retain significant activity following HTST pasteurization and even after UHT treatments (Griffiths *et al.*, 1981; Law *et al.*, 1976; and Cogan, 1977). A D_{150} value (time required for 90% inactivation) of 63 s was determined for the lipase of *P. fluorescens* MC50, a psychrotroph isolated from raw milk (Adams and Brawley, 1981a). Speck and Busta (1968) found that a UHT treatment of 149°C for 3-4 s resulted in less than a 15% reduction in activity of the MC 50 lipase. Although heat resistance decreased as a function of increased purity, Fox and Stepaniak (1983) showed that the purified *P. fluorescens* AFT 36 lipase retained significant activity following UHT pasteurization. In spite of the evidence that these lipases possess the potential to cause hydrolytic rancidity in milk, there has been little documented evidence that it is a common occurrence (Law, 1979; Allen and Joseph, 1985). Law (1979) proposed that the low incidence of bacterial induced rancidity in milk was a result of the inability of the lipases to penetrate the milk fat globule membrane (MFGM) and gain access to the triglycerides, as is the case for the endogenous milk lipase. However, Deeth and

Fitz-Gerald (1983) reported that crude extracts of bacterial lipases were capable of hydrolyzing the triglyceride in intact milk fat globules. Whether the lipases themselves were able to penetrate the MFGM or whether the membrane was disrupted by other enzymes, such as phospholipases, was not determined.

In contrast to milk, hydrolytic rancidity, as a result of heat-stable bacterial lipases, is an important storage defect of high fat dairy products, such as butter and cheese. This may be explained by the observation that the lipases of psychrotrophic bacteria tend to be concentrated in cream and in the curd on coagulation (Downey, 1980). In a study by Kishonti and Sjöström (1970) approximately 80% of a *Pseudomonas* lipase in milk was found to be concentrated in cream, and subsequently in butter. The butter developed rancidity following two days of storage, apparently as a result of the lipase.

The presence of the lipase producing strain of *Pseudomonas AR1* in milk used to produce cheddar cheese before heat treatment, led to high free fatty acid (FFA) levels and to rancidity of the cheese on storage at 12°C for 2-4 months (Law *et al.*, 1976). Lipases from psychrotrophs in milk have also been reported to cause high FFA levels and flavour defects in Swiss cheese (Pinheiro *et al.*, 1985), Dutch cheese (Briessen and Stadhauders, 1975) and Camembert cheese (Dumont *et al.*, 1977).

Heat-resistant proteases of psychrotrophs

Protease producing, psychrotrophic Gram-negative bacteria have commonly

been isolated from raw milk (Speck and Adams, 1976). Adams *et al.* (1975) found that 70-90% of raw milk samples examined contained psychrotrophs capable of producing heat-resistant proteases. As is the case for the lipases, the most active producers of proteases among the psychrotrophs in raw milk are members of the genus *Pseudomonas*, especially the *P. fluorescens* species (Law, 1979; Cogan, 1977).

These enzymes are a major concern to the dairy industry because of their effect on the keeping quality of milk and dairy products. Proteolysis results in irreversible alterations in milk during storage, which include increased nonprotein nitrogen (NPN), off-flavours from bitter peptides, gelation, and changes in viscosity (Visser, 1981).

1.3.3. Characteristics of heat-resistant proteases

Heat stability of proteases

Although the proteases can cause some breakdown of protein in raw milk, their action on stored heat-treated milk and dairy products is considered to be of greater economic importance. Much of the emphasis concerning the spoilage of milk has been directed towards the elimination of heat-resistant bacteria and spores, rather than the levels of psychrotrophic extracellular enzyme which can survive heat treatment (Burton, 1984; Adams *et al.* 1975; Malik and Swanson, 1974). Many of the psychrotroph proteases have the ability to survive conventional HTST pasteurization, as well as UHT sterilization, and appear to be more thermostable than lipases from psychrotrophs (Griffiths *et al.*, 1981).

One of the most extensively studied heat stable proteases is that of the psychrotroph *Pseudomonas* sp. MC60. It has been estimated by Adams *et al.* (1975) that the protease is 4000 times more heat resistant than the spores of *Bacillus stearothermophilus*, with a D value at 140°C of 1.5 min. A temperature of 120°C for 9 min was required to completely inactivate the protease of *P. fluorescens* P26 (Mayerhofer *et al.*, 1973). Protease I of the psychrotroph AFT21 had a D value at 140°C of 0.65 min (Stepaniak and Fox, 1985). Griffiths *et al.* (1981) found that the proteases of 13 *Pseudomonas* species isolated from milk retained 55-65% enzyme activity at 77°C for 15 s, and 20-40% at 140°C for 5 s. Gebre-Egziabher *et al.* (1980a) estimated that the UHT treatment required to completely inactivate the protease of six psychrotrophs would have an adverse effect on the dairy product.

The heat stability of proteases from psychrotrophs is enhanced in milk (Mayerhofer *et al.*, 1973; Barach *et al.*, 1976a). Barach *et al.* (1976a) were able to show that the presence of the chelating agent, EDTA was effective in significantly reducing the heat resistance of the *Pseudomonas* MC60 protease. This suggested that the heat stabilizing effect was because of the divalent metal ions in milk. Both calcium and zinc ions were required for optimal activity, however only calcium ions restored the protective effect of milk against high temperatures (Barach *et al.*, 1976a). Mitchell *et al.* (1986) also found that calcium ions contributed to the heat stability of the extracellular proteases from psychrotrophic pseudomonads.

Barach and Adams (1977) further characterized the MC60 protease, and observed that this protease was similar to the thermophilic protease, thermolysin, with respect to its calcium ion requirements, lack of sulphhydryl groups, molecular weight and hydrophobicity. They suggested that these properties contributed to the enzyme's heat stability by allowing it to be flexible when denatured at high temperatures, then to refold with the formation of salt bridges when the temperature was lowered.

Some heat-resistant psychrotroph proteases have been reported to lose more enzyme activity at low temperatures (45-55°C) than would be predicted (Adams *et al.*, 1975; Gebre-Egziabher *et al.*, 1980b). The protease of *P. fluorescens* AFT36 was unstable at temperatures between 50 and 60°C, losing more than 90% activity at 55°C (Stepaniak and Fox, 1983). Similar results were obtained for the *Pseudomonas* isolate AFT 21 which was more stable at temperatures in excess of 80°C than at 55°C (Stepaniak and Fox, 1985). Barach *et al.* (1978) used the term "low temperature inactivation" (LTI) to describe this phenomenon.

The mechanism for LTI of the heat-stable proteases at 55°C is believed to involve two steps [Barach *et al.*, 1978]. First, the protease undergoes a conformational transition which modifies the enzyme structure, resulting in reversible loss of activity and susceptibility to proteolysis by those proteases which have yet to be denatured. Next, the modified enzyme aggregates with casein micelles to form an enzyme-casein complex, resulting in a reduction of the amount of active protease in the milk. At temperatures of about 60°C or higher the

hydrophobic interactions involved in the enzyme-casein complex are no longer favoured, thus explaining why these proteases are not inactivated to the same extent at higher temperatures.

In an attempt to evaluate the effectiveness of LTI to inactivate heat-resistant protease in milk, West *et al.* (1978) held UHT treated milk at 55°C for 1 h immediately following heat treatment. Protease activity in all milk samples was reduced by approximately 90% with no evidence of spoilage over 300 storage days, while the control milk samples were unacceptable after 3 months. Griffiths *et al.* (1981) were unable to observe such a reduction in activity when a heating step at 55°C for 1 h was carried out immediately after UHT-treatment. Mitchell *et al.* (1986) found that three of the six heat-stable pseudomonad proteases, which they studied, were largely unaffected by a LTI (55°C/1 h) treatment. The level of activity remaining following LTI, coupled with UHT treatment, ranged from 68.3 to 78.1%. Such variability was also noted by Marshall and Marsteller (1981) and thus illustrates the limited value of LTI heat treatments to the dairy industry, for the inactivation of bacterial proteases.

Properties of heat stable proteases

The extracellular heat-resistant proteases of psychrotrophic pseudomonads generally can be classified as metalloproteases based on their inactivation by metal chelating agents such as EDTA and o-phenanthroline (Law, 1979). Reactivation of EDTA-treated proteases has been reported using divalent cations, in particular Ca^{2+} , Zn^{2+} and Co^{2+} (Mitchell *et al.*, 1986; Stepaniak *et al.*, 1982;

Barach *et al.*, 1976a); McKellar and Cholette (1986) were able to show that calcium ions were required during the synthesis of active extracellular protease by *P. fluorescens* B52, indicating that calcium ions are required for structural integrity of the protease as well as for activity.

The temperature optima for the activity of proteases from psychrotrophs range from 25 to 45°C (Alichandrais and Andrews, 1977; Gebre-Egziabher *et al.*, 1980a; Mitchell *et al.*, 1986). The significance of this to the dairy industry is obvious, since UHT-sterilized milk is usually stored without refrigeration for extended periods of time. Even heat-treated or raw milk held at refrigeration temperatures are potentially susceptible to such proteolytic enzymes (Law *et al.*, 1977; Law *et al.*, 1979). The protease of *P. fluorescens* AR11 retained 33% of its maximum activity at 4°C (Alichandrais and Andrews, 1977). The characterization of the proteases of six pseudomonads isolated from raw milk with respect to the effect of temperature gave comparable results (Gebre-Egziabher *et al.*, 1980a).

The pH optima of these enzymes are generally in the range of 6.5 to 8.0 (Mitchell *et al.*, 1986; Fairbairn and Law, 1986a). Significant activity of these proteases has been noted at pH 6.5, the normal pH of milk. The optimum pH for *Pseudomonas* AFT21 protease I was found to be pH 7, with 65 to 75% of its maximum at pH 5.0, the pH of most cultured dairy products.

The molecular weights of the extracellular proteases of *Pseudomonas* spp. range from 38,000 to 50,000 (Stepaniak and Fox, 1985; Fairbairn and Law,

1986a). The amino acid content of these proteases, which have been purified, appears to be similar with respect to high asparagine, glycine, and alanine content, as well as the absence of cystine (Mitchell *et al.*, 1986).

Most of the psychrotrophic pseudomonads which have been studied produce a single extracellular protease (Fairbairn and Law, 1986a). However, Stepaniak and Fox (1985) have isolated two psychrotrophs from raw milk which produce multiple heat-stable proteases. *Pseudomonas* AFT21 produced three extracellular proteases in milk, but the proportions depended on the growth medium and temperature. *P. fluorescens* AFT36 (Stepaniak *et al.*, 1982) produced one major protease and trace amounts of two others.

Protease production by psychrotrophs

There is no clear relationship between the number of psychrotrophs in milk and the production of protease. Law *et al.* (1977) found that *P. fluorescens* AR11 produced no detectable extracellular protease when its numbers reached 8×10^5 cfu/ml but definite activity was observed when the numbers reached 8×10^6 cfu/ml. Protease produced by *P. fluorescens* 22F in milk was detected at the end of the growth phase, when the number of bacteria was 10^7 cfu/ml. (Driessen, 1981). However, when the numbers of these bacteria were increased artificially by the addition of various amounts of milk that had been spoiled by *P. fluorescens* 22F, proteolysis was observed at counts of 5×10^5 cfu/ml. Based on these results, Driesen (1981) concluded that these bacteria accumulated the protease in milk towards the end of their logarithmic growth phase. Protease

production by *P. fluorescens* 32A was observed in the late log and early stationary phases at both 20 and 5°C, when grown in a synthetic medium (McKellar, 1982). Adams *et al.* (1975), showed that *Pseudomonas* MC60 protease was produced throughout the exponential growth at 4°C.

Temperature is an important factor in protease synthesis by psychrotrophs. Peterson and Gunderson (1960) found that a psychrotrophic strain of *P. fluorescens* produced maximum protease at 0°C, with decreased production as the temperature increased to 30°C. Juffs (1976) showed that *P. fluorescens* cultures were more proteolytic at 20°C, and least when grown at 5 or 30°C. He noted that the protease production per unit of cell growth fell as the temperature of incubation was lowered from the optimum. Also the production of protease dropped dramatically at incubation temperatures above the apparent optimum, despite extensive growth. McKellar (1982) observed that maximum enzyme production occurred at 20°C, and that cells grown at 5°C produced up to 55% of the activity found at 20°C. Fairbairn and Law (1986b) reported that *P. fluorescens* NCDO 2085 produced an extracellular protease optimally at 18°C. Protease production per unit of dry weight tended to increase with decreasing temperature. These results supported the hypothesis that psychrotrophic bacteria compensated for decreased enzyme activity at low temperatures by increasing the amount of enzyme produced.

Aeration appeared to be another factor which affected protease production, however the relationship was not clear. Griffiths and Phillips (1984) were able to

significantly decrease protease synthesis by psychrotrophic bacteria growing in milk at 6°C by aeration, while the growth rates and microbial flora were unchanged. Reductions of as much as 50% of the protease produced in non-aerated cultures was reported. A study of two strains of psychrotrophic pseudomonads by TeWhaiti and Fryer (1978) showed some variability with respect to the effect of aeration on protease production, one strain produced a five-fold increase in protease activity, while no increase in protease synthesis was observed when the other strain was grown, with aeration, at 22°C.

Murray *et al.* (1983) showed that nitrogen flushing of milk resulted in the inhibition of protease production by psychrotrophs. Protease activity could not be detected in the nitrogen-flushed milk over an 18 day storage period at 4°C, although the proteolytic psychrotrophs reached a maximum population of 1.8×10^7 cfu/ml. They attributed this inhibition to the low O_2 tension which was achieved in the milk by this process.

In contrast, Rowe and Gilmour (1982) showed that a drop in oxygen tension in simulated milk medium was immediately followed by an increase in the protease synthesis of psychrotrophic *P. fluorescens* strains.

The composition of the growth medium can significantly influence the production of protease by psychrotrophs. The results of McKellar (1982), Juffs (1976) and Mayerhofer *et al.* (1973) have shown that the extracellular protease of *P. fluorescens* appeared to be an inducible enzyme. Juffs (1976) showed that protease synthesis was a function of the amount of organic nitrogen in the

medium while inorganic nitrogen sources were unable to induce enzyme production. Fairbairn and Law (1986b) reported that protease production of *P. fluorescens* NCDO 2085 did not occur under organic nitrogen limiting conditions, while carbon limiting conditions did not inhibit protease synthesis. These results suggested that these extracellular proteases function to supply carbon for growth rather than amino acids for protein synthesis. McKellar and Cholette (1984), however, were able to show that protease production by *P. fluorescens* 32A did occur in mineral salts medium containing a low molecular weight inducer isolated from milk. They suggested that protease production was not a result of nutrient limitation but may be dependent on phosphate concentration.

Amino acids, such as alanine (Amrute and Corpe, 1978) and glutamine (McKellar, 1982) were found to stimulate protease production. Certain carbon compounds can inhibit protease production and glucose appears to cause catabolic repression (Juffs, 1976; McKellar, 1982) in semidefined media. However, in milk the addition of glucose was not an effective inhibitor of protease synthesis.

Protease synthesis from psychrotrophs can be inhibited by chelating agents, such as EDTA and polyphosphates (McKellar and Cholette, 1985; Richardson and TeWhaiti, 1978). Inhibition of protease formation is reversed by divalent cations, particularly Ca^{2+} (McKellar and Cholette, 1985). Amrute and Corpe (1978) reported a requirement for Ca^{2+} in protease production by *P. fluorescens*.

Torrie *et al.* (1983) attempted to determine the role of proteases during the

growth of psychrotrophs in milk, by comparing the growth rates of *P. fluorescens* 32A and a protease deficient mutant of this strain. They found that the extracellular protease was not essential for growth in milk. However, it did provide a selective advantage for the producer organism. The parent outgrew the mutant type at 6°C, yielding a 10-fold greater yield of cells.

Effect of heat-resistant proteases on milk and milk products

The whey proteins, principally β -lactoglobulin and α -lactalbumin, are quite resistant to proteolysis in their native state. As a result, proteolysis in raw milk and dairy products affects primarily with the caseins which comprise approximately 80% of the total milk protein (Fox, 1981).

Studies on the specificity of proteases from psychrotrophs indicate that most attack κ -casein preferentially (Law 1979; Cousin, 1982). The breakdown of κ -casein results in the formation of a para- κ -casein-like fraction, which can destabilize the casein micelles and cause gelation or coagulation of milk similar to the action of rennet (Snoeren *et al.*, 1979). Beta-casein appears to be hydrolyzed more readily than the α -caseins, and their breakdown has been associated with the formation of bitter peptides (Law *et al.*, 1977; Visser, 1981).

Adams *et al.* (1975) reported that the addition of as little as 0.89 enzyme units of the protease of *P. fluorescens* MC80 to UHT sterilized milk caused rapid spoilage (14-32 days). Law *et al.* (1977) isolated a heat-stable protease from *P. fluorescens* which gelled UHT-sterilized milk. White and Marshall

(1973) noted a significant reduction in the shelf-life and quality of cottage and cheddar cheese when the products were made from milk inoculated with *P. fluorescens* P26, or its heat-stable protease. Jackman *et al.* (1985) demonstrated that milk incubated in the presence of the protease of *Pseudomonas* T25 had an increased clotting time with chymosin, resulting in a weak curd.

McKellar (1981) studied the relationship between off-flavour development in UHT and pasteurized milk, and proteolysis. He found a linear relationship between proteolysis in the milk samples and the amount of psychrotroph protease which was added. Bitter off-flavours also increased, but not in a strictly linear fashion. Pasteurized milk appeared to be more resistant to proteolysis than UHT milk. The use of high temperatures during UHT treatment may result in the exposure of new substrate sites on the casein molecules.

Adams *et al.* (1976) demonstrated that proteolysis by a psychrotrophic *Pseudomonas* species predisposed the caseins and whey proteins to damage by UHT-treatment, resulting in the coagulation of milk upon heating. The adverse effect of proteolytic psychrotrophs on the heat-stability of pasteurized milk was also reported by Cousin and Marth (1977a).

Proteolytic enzymes of psychrotrophs may also have a beneficial effect on some cultured dairy products by increasing acid production by lactic streptococci (Cousin and Marth, 1977a) and lactobacilli (Cousin and Marth, 1977b). Milk precultured with psychrotrophic bacteria were better substrates than uncultured

milk for lactic acid production by starter cultures. Cousin and Marth (1977b) attributed the increased acid production to products of protein degradation which had become available to the lactic acid bacteria by proteolysis.

1.4. Objectives of this study

The ubiquitous nature of psychrotrophs has made it practically impossible to prevent their contamination of raw milk. Consequently the extracellular enzymes they produce, in particular heat-stable proteases, are of concern to the dairy industry. This study was undertaken to investigate such enzymes with the following objectives.

1. To develop a simple and rapid method to purify the heat-stable extracellular proteases of psychrotrophic pseudomonads.

2. To characterize proteases produced by bacteria isolated in Newfoundland (Nfld.) on the basis of the biochemical and physicochemical properties of the former.

3. To elucidate the mechanism of heat-resistance of the proteases from psychrotrophs using circular dichroism (CD)-spectra.

4. To determine the antigenic relatedness between those selected proteases from bacteria isolated in Nfld., as well as those from Ontario, British Columbia, Ireland, U.S.A., Netherlands and Australia.

5. To compare the psychrotrophic pseudomonads from different geographical regions on the basis of the mole% G+C content of their DNAs.

6. To develop a sensitive enzyme-linked immunosorbent assay (ELISA) for the detection of psychrotrophic proteases in milk.

Chapter 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

The following chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.): acrylamide, 5-aminosalicylic acid, ammonium persulfate, antipain, α -N-benzoyl-DL-arginine-p-nitroanilide (BAPA), blue dextran, bovine serum albumin, bromophenol blue, catalase, collagen, Coomassie Brilliant Blue R250, L-cysteine elastin-orcin, ethylenediamine tetraacetic acid (EDTA), Folin-Ciocalteu reagent, glucose-6-phosphate, hemoglobin, horseradish peroxidase, imidazole, lysozyme, 2-mercaptoethanol, N,N'-methylene-bis-acrylamide, myoglobin, β -nicotinamide adenine dinucleotide phosphate (NADP⁺), p-nitrophenylphosphate, ovalbumin, α -phenanthroline, phenylmethylsulfonyl fluoride (PMSF), ribonuclease A, sodium dodecyl sulphate (SDS), soybean trypsin inhibitor, p-toluenesulphonyl-L-arginine methyl ester, trichloroacetic acid (TCA), tris-(hydroxymethyl)-amino methane (Trizma base), tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), and trypsin.

The following were purchased from Chemical Dynamics Corp. (South Plainfield, N.J.): α -casein, β -casein, γ -casein, α -lactalbumin, β -lactoglobulin,

benzyloxycarbonyl-(CBZ)-L-tyrosyl-L-serine, CBZ-L-tyrosyl-L-alanine, CBZ-L-tyrosyl-L-threonine, CBZ-L-tyrosyl-L-leucine and CBZ-L-tryptophanyl-L-alanine.

The following were obtained from BDH Chemicals Can. Ltd. (Poole, England): 2,2'-bipyridyl, casein powder (soluble), *p*-chloromercuribenzoate, *N*-ethylmaleimide and 8-hydroxyquinoline.

Dansyl chloride and Nin-Sol (ninhydrin reagent) were the products of Pierce Chemical Co. (Rockford, Ill). Freund Adjuvant, noble agar, tryptic soy agar and tryptic soy broth were obtained from Difco Laboratories (Detroit, Mich.). *N,N,N,N*-tetramethylethylene diamine (TEMED) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) low molecular weight standards were purchased from Bio-Rad Laboratories (Mississauga, Ont.), while 1-fluoro-2,4-dinitrobenzene was a product of Eastman Kodak Co. (Rochester, N.Y.).

All other chemicals used in this study were of analytical grade.

2.1.2. Organisms and cultivation

Samples of raw milk from the refrigeration storage containers of a local Newfoundland dairy were plated on Difco tryptic soy agar (TSA) containing 2% milk powder. The inoculated TSA plates were incubated for 10 days at 7°C. Those colonies which exhibited proteolytic activity, as indicated by clearing of the surrounding medium, were selected and purified by successive transfers on TSA plates. Of the twenty-eight cultures obtained, six of the most proteolytic isolates were chosen for further study based on their production of extracellular proteases

capable of surviving a heat treatment of 90°C for 10 min. These isolates were subsequently identified as pseudomonads and designated, T8, T13, T16, T20, T22 and T26 (Patel *et al.*, 1983).

Psychrotrophic bacterial isolates of milk origin were also obtained from British Columbia (BC 1-1, BC 2-2), Ontario (13, 15, 49, 32A, and 33), Ireland (AFT 7, AFT 21 and AFT 36), U.S.A. (M5), Netherlands (22F) and Australia (240).

Pseudomonas fluorescens ATCC 15456 was also used in this study.

2.2. Methods

2.2.1. Production of protease

Six 500-ml culture flasks, each containing a total volume of 150-ml tryptic soy broth (TSB) and 2% milk powder were inoculated with 2 ml of 18-24 h broth cultures. All of the flasks were incubated at 25°C for 4-5 days without shaking. The bacterial cells were pelleted by centrifugation at 10,000 x g for 10 min. The supernatant solutions were filtered using 0.22 µm Millipore filters and transferred to dialysis tubing (Spectrapor, 12,000 m.w. exclusion) and dialyzed against 20 mM Tris-HCl buffer (pH 7.5) at 5°C for 24 h. This was the source of the crude enzyme extract.

Protease assay using modified Hull's method

This procedure was a modification of Hull's-tyrosine method (Hull, 1947).

The substrate, 1% soluble casein in deionized water, and the enzyme samples were extensively dialyzed against 20 mM Tris-HCl buffer, pH 7.5, overnight at 5°C. The reaction mixture consisted of 0.5 ml of substrate, enzyme extract (0.1-0.5 ml) and 100 mM Tris-HCl buffer pH 7.5 to a total volume of 2.0 ml. The reaction mixture was incubated at 25°C for 20 min in a temperature regulated waterbath. The reaction was terminated by the addition of 1.0 ml of 5% trichloroacetic acid (TCA) to each tube and cooling in ice for 30 min. The precipitated proteins were removed by centrifugation at $10,000 \times g$ for 10 min using a Sorvall Superspeed Refrigerated Centrifuge. The TCA soluble peptides and amino acids in the supernatant were measured by the absorbance at 280 nm. The controls were prepared in the same manner except that the substrate was added following the addition of the 5% TCA.

One enzyme unit was defined as the amount of enzyme extract that released 1 μ mole of tyrosine equivalence per min per ml under the standard assay conditions. The absorbance of 1 μ mole of tyrosine at 280 nm under standard assay conditions was determined to be 0.41. Specific activity was defined as enzyme units per mg of protein. Unless stated otherwise, this was the standard protease assay method.

Protein determination

Protein concentration was determined by a modification of the procedure of Lowry *et al.* (1951), using bovine serum albumin (BSA) as the standard protein. The reagents used were prepared as described in Appendix B.

The sample was made up to a volume of 0.2 ml with deionized water, followed by the addition of 1.0 ml of the alkaline copper tartrate reagent (Solution III). The mixture was incubated at 25°C for 15 min. Then, 0.1 ml of Folin-Ciocalteu reagent (Solution IV) was added and incubated at 25°C for 20 min. The absorbancies were measured at 600 nm using a Shimadzu UV-Visible Recording Spectrophotometer (UV-260). The blank consisted of 0.2 ml deionized water. A standard curve was prepared using of 10 μ g to 70 μ g of BSA.

2.2.2. Enzyme purification by affinity chromatography

CBZ-DL-phenylalanine-TETA Sepharose 4B

Crude enzyme extract (500 ml) was lyophilized and the dry residue was dissolved in 50 ml of 25 mM sodium acetate buffer (pH 5.8) containing 0.1 M NaCl and 0.01 M CaCl_2 and dialyzed against 5 l acetate buffer overnight at 5°C. Proteins which had come out of solution during dialysis were removed by centrifugation (10,000 rpm for 10 min). The concentrated extract was applied to an affinity column of carbobenzoxy-DL-phenylalanine-triethylene tetraamine Sepharose 4B (Pierce Chemical Co., Rockford, Ill.). The column (0.8 x 25 cm) containing 10 ml of the affinity material had been washed with 200 ml of the 25 mM sodium acetate buffer. The unbound protein was eluted with 400 ml of the sodium acetate buffer and the bound protein was eluted with 100 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl and 0.01 M CaCl_2 . Approximately 3-ml fractions were collected and the protein content was monitored at 280-nm with the model UV-260 spectrophotometer. The proteolytic activities in the fractions were determined by the modified Hull's method. Appropriate fractions were

pooled and concentrated to 3 ml by ultrafiltration in an ultrafiltration cell with a UM-10 membrane at 40 psi (Amicon Corp., Oakville, Ont.). This concentrated protease was then dialyzed against 20 mM Tris-HCl buffer (pH 7.5) at 5°C for 18 h.

Hemoglobin-sepharose affinity chromatography

An attempt was made to purify the protease by using cyanogen bromide activated Sepharose 4B covalently linked with hemoglobin. The affinity column material was prepared by the method of Chua and Bushuk (1969) as described in Appendix A.

The affinity material was equilibrated by washing with 200 ml of 50 mM sodium acetate buffer (pH 5.4). The crude lyophilized enzyme extract (5 ml), which had been dialyzed against the acetate buffer overnight at 5°C, was applied to the hemoglobin-Sepharose column (20 x 1 cm). The unbound protein was eluted with 400 ml of the acetate buffer and the bound protein was released with 100 mM Tris-HCl buffer, pH 7.5. The protein content of each 3 ml fraction was monitored at 280 nm and the protease activity determined by the standard assay method.

The protease did bind to the hemoglobin-Sepharose column material, and was eluted with the Tris-HCl buffer. However, two protein peaks which overlapped extensively were detected. Most of the protease activity was associated with the second, while the fractions of the first protein peak contained very little protease activity.

2.2.3. Effect of assay conditions on protease activity

Effect of pH on protease activity

Each of the six partially purified proteases were assayed by the standard protease assay method (modified Hull's method) except that the pH of the 100 mM Tris-HCl buffer was varied, the pH values were 7.0, 7.5, 8.0, and 9.0. For pH 5.0, 6.0 and 7.0, 100 mM citrate-phosphate buffer was used.

Effect of incubation temperature and determination of activation energies

Crude enzyme extracts were assayed by the standard assay method at various incubation temperatures (0 to 40°C) in a temperature regulated waterbath containing ethylene glycol, using 1% soluble casein as the substrate. The substrate was maintained at the appropriate incubation temperature before addition to the reaction mixture.

The activation energies (E_a) for the protease catalyzed casein hydrolysis were determined using the Arrhenius equation, which relates reaction velocity to absolute temperature. The observed activation energy was determined by multiplying the negative slope by a correlation coefficient of $2.303 \times R$, where R represents the gas constant ($R=1.9$).

2.2.4. Effect of protease inhibitors and activators

Inactivation of proteases by EDTA

Crude enzyme extracts were dialyzed overnight at 4°C against 20 mM Tris-HCl buffer (pH 7.5) containing 100 mM EDTA. Almost a complete loss of protease activity resulted from this treatment. The EDTA was removed by extensive dialysis of the inactivated proteases against 20 mM Tris-HCl buffer (pH 7.5).

Reactivation of EDTA-treated protease with metal ions

The EDTA-treated proteases (apoenzymes) were preincubated for 20 min at 5°C, at different concentrations (4 and 6 μ mol per assay) of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ or $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$. The extent of reactivation of the apoenzymes by the metal ions was determined under standard assay conditions and expressed as a percentage of the protease activity of the non-EDTA treated proteases.

Effect of metal ions on protease activity

The effect of added metal ions on protease activity was determined using the standard assay method. The metal ions ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 2\text{H}_2\text{O}$, $\text{HgCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) were dissolved in 100 mM Tris-HCl buffer (pH 7.5) and added to the assay mixture at final concentrations of 1 and 2 mM. A preincubation step of 5°C for 20 min, before the

addition of the substrate, was included. The activity in the presence of each metal ion was expressed as a percentage of the protease activity obtained without added metal ions.

Determination of metal ion content

Purified enzyme solutions were dialyzed extensively against deionized distilled water. The enzymes (0.3 mg/ml) were analyzed by atomic absorption spectrometry, using single element lamps, at the most sensitive line for each element (Ca, Mg, Zn and Mn).

Effect of protease inhibitors

The effect of protease inhibitors on the activity of protease T16 was determined by including various inhibitors in the standard reaction mixture. The inhibitors used were, *p*-chloromercuribenzoate, *o*-phenanthroline, 8-hydroxyquinoline, 2,2'-bipyridyl, ethylenediaminetetraacetic acid (EDTA), L-cysteine, N-ethylmaleimide, pepstatin, antipain, soybean trypsin inhibitor, and phenylmethylsulfonyl fluoride (PMSF). Those compounds which were insoluble in deionized water were dissolved in a minimum amount of dimethyl sulfoxide (DMSO). The final amounts of the inhibitors, per assay, ranged from 2 to 10 μ moles. A preincubation step which consisted of maintaining the mixture of enzyme, buffer, and inhibitor at 5°C for 20 min was included, before the addition of the substrate. The percent inhibition was based on a control assay without the presence of inhibitors.

2.2.5. Thermal stability studies

Heat stability of the T16 protease

Heat-sealed glass ampoules (2 ml capacity) containing 0.4 ml of T16 enzyme extract were completely immersed in a waterbath at 50°C. Following a heat-up time of 60 sec ampoules were removed at different time intervals (0.5-10.0 min) and immediately placed in ice and allowed to cool. The enzyme extracts were removed from the ampoules and centrifuged at $10,000 \times g$ for 10 min to remove any denatured proteins. The supernatant was then assayed for protease activity using the standard assay method.

The above procedure was repeated at different temperatures (90, 120 and 150°C). For the 120 and 150°C temperature-treatments an oil-bath was used.

Heat stability of the protease in the presence of metal ions

Different amounts of CaCl_2 or MgCl_2 (0-40 μmole) were incubated with 0.2 ml of the partially purified T16 protease in 100 mM Tris-HCl, pH 7.5 (total vol. 1.5 ml), for 20 min at 0-5°C. The reaction mixture was then heated for 10 min at 90°C. After cooling to 25°C, the standard enzyme assay was performed. Unheated controls with added metal ions were also prepared and they represented 100% activity.

A similar experiment was performed to determine whether the addition of metal ions to the protease, following the heat treatment, would restore any of the

lost activity. After heating at 90°C for 10 min, and cooling to 25°C, CaCl_2 or MgCl_2 (0-20 μmole) was added to the T16 samples and incubated for 1 h at 0-5°C. Again the non-heated enzyme was considered to represent 100% activity.

To investigate the possibility of reactivation of heat treated proteases with time, the partially purified proteases of isolates T13, T16 and T20 were stored on ice for 24 h immediately after heat-treatment (90°C for 10 min). The protease activities were compared following the cold storage.

Heat stability of T16 protease in the presence of lactose

Partially purified T16 protease (0.2 ml) was incubated in the presence of various amounts of lactose (0-5 mg in 100 mM Tris-HCl, pH 7.5) for 20 min at 0-5°C. The mixture (total vol. 2.0 ml) was heated at 90°C for 10 min, and then cooled in ice. The protease activity was then determined by the standard assay method. Unheated enzyme controls containing lactose were also prepared and their activity represented 100%.

2.2.6. Physicochemical properties of proteases

Determination of isoelectric points by chromatofocusing

Chromatofocusing with polybuffer and polybuffer exchanger allows the separation of proteins according to their isoelectric points (pI) in a column. (Lampson and Tytell, 1965). The isoelectric points of the six proteases were determined using a chromatofocusing kit (Pharmacia Fine Chemicals, Montreal, Canada). A column (1 x 30 cm; 20 ml bed volume) was equilibrated with a start-

buffer (25 mM Imidazole-HCl, pH 7.4). The crude enzyme extracts (10 mg protein in 5 ml) were dialyzed against the start buffer and applied to the column which had been washed with 250 ml of the start buffer. Protein in the fractions was monitored at 280 nm. Polybuffer 74 diluted 1:8 with distilled water and adjusted to pH 4.0 with 100 mM HCl was used to elute the bound proteins. The pI of a sample was taken as the pH of the fraction at which maximum protease activity was detected. The column was maintained at 5°C at all times.

Amino acid analysis

Amino acid analysis was performed after hydrolysis in 6N HCl at 110°C for 24, 48 and 72 h, in a Beckman model 121 MB amino acid analyzer. A single column of Beckman AA10 resin was used with a three buffer lithium system as described in Beckman Technical Bulletin 121MB-TB-017. The amino acid composition was estimated as follows; (1) for those amino acids with constant values for all hydrolysis times the arithmetic mean was used (2) for those which increased with time of hydrolysis the maximum value obtained was used (3) for those which decreased with time of hydrolysis the values were extrapolated to zero time.

Cysteine and methionine were determined by performic acid hydrolysis (Bailey, 1967). Performic acid (2 ml) was added to 1 mg of protein and stored at 5°C overnight. Distilled water (8 ml) was then added, the mixture was lyophilized and then hydrolyzed in 6 N HCl for 24 h. Cysteine and methionine were then determined by amino acid analysis as cysteic acid and methionine

sulfone. Tryptophan was determined after hydrolysis of the protein in 3 N mercaptoethanesulfonic acid as described by Penke *et al.* (1974).

Estimation of purity and molecular weights of proteases

Polyacrylamide gel electrophoresis

Disc-gel electrophoresis was performed according to the method of Davis (1964) to determine the homogeneity of the purified protease. The 7.5% polyacrylamide gels were prepared as described in Appendix C. Electrophoresis was carried out using a model 150A gel electrophoresis cell (Bio-Rad). The solution in the cell was 200 mM Tris-glycine buffer pH 8.3. Cold water was circulated through the chamber to maintain a low temperature, and a constant current of 2 mA per gel was applied using a Buchler 3-1500 power supply (Buchler Instruments Inc., Fort Lee, New Jersey). Bromophenol blue (0.05% in water) was used as the tracking dye. Electrophoresis was judged to be complete when the tracking dye was approximately 1 cm from the bottom of the gel. The gels were removed from the tubes and were fixed in 10 ml of 12% TCA for 1 h. The gels were transferred from the TCA solution and stained overnight using 0.25% Coomassie Brilliant Blue R250. The gels were destained in 7.5% acetic acid with 5% methanol using a power destainer.

SDS polyacrylamide gel electrophoresis

The relative molecular weight of the purified T16 protease was determined by gel electrophoresis using the Weber and Osborn (1960) method. The purified

protein (40-80 μ g) was reduced by boiling for 2-3 min in the presence of 0.1% sodium dodecyl sulphate and 0.1% 2-mercaptoethanol. The protein was applied to 10% polyacrylamide gels (Appendix D) and electrophoresis was conducted at a constant current of 2 mA per gel in 10 mM sodium phosphate buffer (pH 7.0).

The gels were fixed in 12% TCA, and stained and destained as described for the Davis PAGE gels. The mobilities of the proteins were determined by the equation:

$$\text{Mobility} = \frac{\text{migration of protein}}{\text{length gel destained}} \times \frac{\text{length gel before staining}}{\text{migration of dye}}$$

The mobility was plotted against known molecular weights of standard proteins expressed on a semi-logarithmic scale.

The molecular weight standards were included for each run and consisted of the following proteins: phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Gel filtration of proteases

The molecular weights of the proteases were also determined by gel filtration according to the procedure described by Andrews (1964). Crude lyophilized enzyme extracts (10 mg protein/5 ml) were applied to a calibrated Sephadex G-150 column (250 ml bed volume, 4 x 50 cm); which had been equilibrated with Tris-HCl buffer (pH 7.5). The column was washed with the same

buffer and 3 ml fractions were collected. Fractions were assayed for protein content and enzyme activity. Proteins of known molecular weight used as standards were BSA (66,200), ovalbumin (45,000), lysozyme (14,400), trypsin (23,000), catalase (60,000), and cytochrome c (11,700).

Ultracentrifugation of protease T16

The sedimentation velocity of purified T16 enzyme was determined from the Schlieren patterns obtained when the solution of enzyme (1.5 mg/ml) was centrifuged at 20°C and 60,000 rpm in a Spinco Model E Ultracentrifuge.

A single symmetrical peak was observed in the Schlieren profile of the sedimenting protein. The sedimentation coefficient (S-value) was calculated using the sedimentation data.

Difference Index (DI)

The difference index (Metzger *et al.*, 1968) has been used to predict whether two proteins are related based on their amino acid compositions. D.I. values less than 10 indicate relatedness between a pair of proteins and values greater than 26.8 indicate unrelatedness (Woodward, 1978). Two proteins were compared by determining the difference in the fractional content of each amino acid, obtaining the sum of the absolute value of these differences and multiplying that sum by 50.

N-Terminal amino acid analysis

Labelling of the N-terminal amino acid of the T13, T16 and T20 proteases, was carried out using the dansyl chloride (DNS-Cl) technique of Woods and Wang (1967). To an enzyme sample (100-200 μ g of protein) dissolved in 50 μ l of 500mM sodium carbonate (pH 7.0) containing 1% SDS was added one-half volume of DNS-Cl solution (5 μ g/ml) in acetone and incubated at 37°C for 3 h. The protein was precipitated with 5 volumes of 10% TCA and pelleted by centrifugation at 10,000 x g for 10 min using a Sorvall Superspeed Centrifuge. The precipitate was washed with 2 ml of 1 M HCl to remove the soluble dansylic acid and dried in a dessicator. The dansylated protein was hydrolyzed in 6 N HCl at 110°C for 10 h. The hydrolyzed protein was dried in a dessicator over potassium hydroxide. The residue was dissolved in about 10 μ l of ethyl acetate. The separation and identification of the dansylated amino acid was carried out using the polyamide thin layer chromatography method of Hartley (1970). Standard dansylated amino acids were included for the identification of the dansylated amino acids in the sample.

Amino sugar determination

The amino sugar content of the T16 protease was determined by the method of Black *et al.* (1970). The protein (0.5 mg) was hydrolyzed (under vacuum) with 4 N HCl at 100°C for 8 h. The HCl was removed in a vacuum over sodium hydroxide and the dried sample reconstituted with 200 mM sodium citrate buffer (pH 2.2). Analysis was performed on a Beckman W2 resin bed at 65°C. The

elution buffer was 400 mM sodium citrate pH 4.1. The detection agent was ninhydrin.

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is a technique which can separate proteins on the basis of their relative overall hydrophobicities, as indicated by the differing strengths of their hydrophobic interactions with an uncharged chromatography material which contains nonpolar (hydrophobic) groups.

The overall hydrophobicities of the proteases were compared based on the polarity and ionic strength of the eluent required to desorb the protein from the Octyl-Sepharose CL-4B gel (Pharmacia Chemicals, Montréal, Quebec). The crude enzyme extract (5 mg protein/ml) was dialyzed against the start buffer, 10 mM sodium phosphate buffer, pH 6.8, containing 25% ammonium sulphate. The enzyme extract (5 ml) was applied to the Octyl-Sepharose CL-4B column which had been equilibrated with the start buffer. The column (20 x 1 cm) contained 8 ml of the gel material and was maintained at 5°C. The unbound protein was eluted with 200 ml of the start buffer. The bound protein was desorbed from the column by decreasing the ionic strength and increasing the concentration of ethylene glycol in the eluent with a linear gradient. The gradient was created by mixing 100 ml of 25% ethylene glycol in phosphate buffer, to 100 ml of the start buffer (25% ammonium sulphate in phosphate buffer) at a rate of 0.5 ml per min using a gradient mixer (LKB-Product AB, Stockholm Sweden) with constant

stirring. Approximately 2 ml fractions were collected and the protein which was released was monitored at 280 nm. The proteolytic activity in the fractions was determined by the standard protease assay method. The relative hydrophobicities of the proteases were expressed as the concentration of ammonium sulphate and ethylene glycol required to release the enzymes from the Octyl-Sepharose CL-4B column. Two standard proteins, catalase and myoglobin, were treated in the same manner. The elution of these proteins was monitored by the change in absorbance at 280 nm.

2.2.7. Protease specificity

Protease specificity to protein substrates

The enzyme extracts were assayed for activity using the ninhydrin protease assay method (Reimerdes and Klostermeyer, 1976) with a total of nine different protein substrates. The substrates used were α -casein, β -casein, γ -casein, κ -casein, α -lactalbumin, β -lactoglobulin, bovine serum albumin (BSA), ovalbumin, and hemoglobin. Each substrate was dissolved in 100 mM Tris-HCl buffer (pH 7.5) to a concentration of 2.5%. The results were expressed as a percentage of the activity obtained when soluble casein was used as the substrate.

Specificity of protease to peptide substrates

The hydrolytic activity of the T16 protease against a number of synthetic peptides (z-tyr-ala, z-trp-ala, z-tyr-ser, z-tyr-thr, and z-tyr-leu) and polypeptides (B-ala-pro-tyr-gly-NHMe, B-ile-pro-tyr-ala-NHMe, B-leu-pro-tyr-ala-NHMe, and B-

ala-pro-tyr-ala-NHMe) was determined. The peptide solutions (2 mM) were prepared by dissolving the peptides in deionized water and adjusting the pH to 7.0 with 1 N NaOH. The reaction mixture was made up of 0.5 ml of the peptide solution, 1.0 ml of Tris-HCl buffer (pH 7.5) and 0.2 ml of enzyme. The reaction mixture was incubated at 25°C for 18 h. The reaction was stopped by the addition of an equal volume of ninhydrin reagent and the mixture was heated at 100°C in a waterbath for 15 min. After cooling to room temperature, 5.0 ml of 60% ethanol was added and the absorbance measured at 570 nm using the model UV-260 spectrophotometer.

Elastase activity

To determine if the protease exhibited elastase activity, the ability of these enzymes to hydrolyze elastin was examined. The reaction mixture consisted of 100 µg of purified protease, 20 mg of elastin-orcin (Sigma) and 200 mM Tris-HCl, pH 7.5, made up to a total volume of 2.0 ml. The assay mixture was incubated in a shaker waterbath at 37°C for 4 h with shaking (100 rpm). The reaction was stopped by centrifuging the reaction mixture for 5 min in an Eppendorf microfuge (12,000 rpm) at room temperature to remove the unhydrolyzed substrate. The absorbance of the supernatant was measured at 570 nm. Appropriate controls without substrate or enzyme were also included.

Collagenase activity

The collagenase activity of these proteases was determined by the ninhydrin method with insoluble collagen as the substrate (Worthington, 1972).

The reaction mixture consisted of purified protease (10 and 20 μg), 25 mg of insoluble collagen (Sigma) and 4 ml of 50 mM TES buffer containing 0.004% CaCl_2 pH 7.5, in 25 ml Erlenmyer flasks. The flasks were incubated in a shaker waterbath at 35°C for 7 h. The unhydrolyzed collagen was removed by centrifugation using an Eppendorf microfuge (12,000 rpm) for 5 min. To 0.5 ml of the supernatant was added 1.0 ml of ninhydrin reagent which was then heated in sealed tubes for 15-20 min in a waterbath at 100°C . The tubes were cooled to room temperature followed by the addition of 1.5 ml of 60% ethanol. The absorbance of the solutions was read at 570 nm.

Hydrolysis of BAPA

The hydrolysis of BAPA, (α -N-benzoyl-DL-arginine-p-nitroanilide) was carried out according to the method of Erlanger *et al.* (1961).

Substrate Preparation: 1 mM BAPA was prepared by dissolving 43.5 mg of BAPA in 1.0 ml of dimethyl sulfoxide (DMSO) and 0.1 ml portions were made up to 10 ml with 50 mM Tris-HCl, pH 8.2 containing 20 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

Assay Procedure: Purified T16 protease (50 μg), 0.5 ml, was added to various concentrations of the BAPA substrate solution in a quartz cuvette (3 ml capacity) at room temperature. The change in absorbance at 410 nm was measured continuously for 10 min using the model UV-260 spectrophotometer.

Hydrolysis of TAME

The hydrolysis of the synthetic substrate TAME, (p-toluenesulphonyl-L-arginine methyl ester) was determined by the method described by Hummel (1959).

Substrate preparation: 37.9 mg of TAME was dissolved in 10 ml of assay buffer (50 mM Tris-HCl, pH 7.5).

Assay procedure: The substrate solution (0.1-0.5 ml) was added to the assay buffer followed by the T18 enzyme (50 and 100 μ g) to give a final volume of 3.0 ml. The reaction was carried out in a quartz cuvette (3 ml capacity) at 25°C and the absorbance measured continuously at 247 nm using a Shimadzu UV-Visible Recording Spectrophotometer Model UV 260.

Ninhydrin method

The procedure was the same as the modified Hull's method except that the free amino groups were detected using the ninhydrin reagent. The ninhydrin reagent (0.5 ml) was added to 0.5 ml of the test solution. The tubes were sealed and the mixture heated at 100°C for 15 min in a temperature regulated waterbath. After cooling to room temperature, 5 ml of 60% ethanol was added and the absorbance was measured at 570 nm.

pH-Stat titration method

The protease activity was measured using an automatic Metrohm pH-stat/end point titrator (Brinkman Instruments, Rexdale Ont.) equipped with a

chart recorder. The protease activity was expressed as the amount of base (KOH) required to titrate the free amino groups which were generated by the hydrolysis of the peptide bonds of the substrate by the protease.

Procedure: 5 ml of 2% soluble casein (pH 8.0) was equilibrated at 25°C using a circulating waterbath. Once a flat baseline was obtained for 10 min the enzyme was added (40-100 μ g in 100 μ l). The base uptake was recorded for 20 min with the titrator set at pH 8.0 and the standardized 100 mM KOH solution in the titrator.

2.2.8. Circular dichroism (CD) spectra of the T16 protease

The effect of temperature on the secondary structure of the purified T16 protease was investigated by comparing the CD spectrum of the enzyme at different temperatures. The CD measurements were obtained with a Jasco J-20 A Spectropolarimeter. To the sample cell (0.1 cm path length) was added 100 μ l (40 μ g) of the T16 protease which had been dialyzed against 20 mM Tris-HCl buffer (pH 7.5). The sample was scanned at wavelengths from 245 to 195 nm at a rate of 50 nm/min, with a sensitivity value of 2, a chart speed of 10 cm/min and a sampling time of 1 ms. The temperature of the sample cell was adjusted using a temperature regulated waterbath. The temperature ranged from 25 to 95°C. The spectrum was initially determined at 5°C.

2.2.9. Localization of active protease and protease precursor

Localization of the active protease and inactive precursor of the T16 isolate was carried out by selective fractionation of the bacterial cells by spheroplast formation and mechanical breakage.

Spheroplast formation

The method used to produce spheroplasts was the procedure described by Jensen *et al.* (1980). A one-litre Erlenmeyer flask, containing 300 ml of low phosphate medium (Appendix E) and 1% soluble casein was inoculated with an 18 h culture of isolate T16, and incubated at 25°C (without shaking) for 72 h. The cells were spun down by centrifugation at 10,000 x g for 10 min at 4°C. The supernatant (Fraction I) was dialyzed against 20 mM Tris-HCl buffer (pH 7.5), overnight at 4°C and then frozen. The cells were washed with 10 ml of 200 mM MgCl₂ in 100 mM Tris-HCl, pH 8.4. This washing step was repeated three times. The supernatant from the final wash was assayed for proteolytic activity to ensure that no active extracellular protease remained. The cells were suspended in 10 mM Tris-HCl (pH 8.4) containing 10 mM MgCl₂ (spheroplast formation step). The spheroplasts were centrifuged (10,000 x g for 10 min) and the supernatant (Fraction II) was frozen. The spheroplasts were washed three times with 10 mM Tris-HCl, pH 8.4, with 10 mM MgCl₂. Spheroplast formation was confirmed by monitoring each fraction for periplasmic specific (alkaline phosphatase) and cytoplasmic specific (glucose-6-phosphate dehydrogenase) marker enzymes.

The spheroplast pellet (3 ml) was put in an X-Press cell (Ab Biotec, Stockholm, Sweden) and frozen at -30°C , by submerging the cell in a mixture of 60% ethanol and dry ice. The spheroplasts were broken using a hydraulic press at 10,000 psi. The ruptured spheroplasts were suspended in a minimum amount of 10 mM Tris-HCl (pH 8.4) with 10 mM MgCl_2 , and examined microscopically to ensure that breakage had been accomplished. The broken spheroplast suspension was then centrifuged twice at $3,000 \times g$ for 10 min at 4°C to remove any unbroken cells. Centrifugation of the broken cell suspension for 1 h at $105,000 \times g$ in a Sorvall Ultracentrifuge OTD-50 yielded a pellet of cell envelope (Fraction III) and the supernatant containing the cytoplasmic contents (Fraction IV). The control consisted of ruptured cells which had not been subjected to osmotic shock treatment.

Alkaline phosphatase assay

Activity of the periplasmic enzyme alkaline phosphatase was determined by the method of Garin and Levinthal (1960). The reaction mixture consisted of 0.5 ml of the sample fraction in 1.0 ml of Tris-HCl buffer (pH 8.0), followed by 0.5 ml of substrate (40 mM *p*-nitrophenylphosphate). The reaction mixture was incubated at 37°C for 1 h. The amount of *p*-nitrophenol released was detected spectrophotometrically at 420 nm.

Glucose-6-phosphate dehydrogenase assay

The cytoplasmic enzyme, glucose-6-phosphate dehydrogenase was measured

by following the reduction of NADP^+ as determined by the change in absorbance at 340 nm (Malamy and Horecker, 1964). The reaction mixture consisted of 0.1 ml of 100 mM glucose-6-phosphate, 0.1 ml of 6 mM NADP^+ , 0.1 ml of sample, made up to a total volume of 3.0 ml with 55 mM Tris-HCl buffer, pH 7.8. The reaction mixture was incubated at 30°C for 15 min. One enzyme unit reduced 1 μ mole of NADP^+ per min at 30°C.

Detection of protease precursor (proprotease)

The presence of both active and inactive forms of the protease in each fraction was determined by the formation of a precipitin band by the Ouchterlony immunodiffusion test using anti-T16 protease IgG as described in section 2.2.11.

Proteolytic activation of proprotease

An attempt was made to activate the proprotease in Fraction II by limited proteolysis with both active purified T16 protease and trypsin.

Fraction II, 100 μ l; 10 μ g of T16 protease or trypsin; and 100 mM Tris-HCl pH 7.5 made up to a final volume of 1.0 ml, were incubated at 35°C for 15 min. The substrate, 0.5 ml of 1% soluble casein was added and the protease activity was determined after an incubation time of 20 min at 35°C. This was done by measuring the release of TCA-soluble amino acids at 280 nm.

2.2.10. Mole percent G+C determination of psychrotrophic strains

The stability of DNA to heat denaturation has been correlated with the content of guanine and cytosine (G and C) base pairs. The mole percent G+C can be estimated by determining the midpoint of the melting temperature of a DNA sample i.e. the T_M value.

The T_M value of each DNA sample was determined automatically using a programmable Beckman T_M Analysis System (Beckman Instruments, Inc., Fullerton, Ca.). This consisted of a six-position temperature controlled sample holder, a T_M compuset module and a DU-8-Visible computing Spectrophotometer with digital plotter.

Procedure: The DNAs of 19 of the psychrotrophic strains were purified by the method of Marmur (1961) as described in Appendix F. A sample volume of 250 μ l was added to each of the six sample cells. The absorbance was measured at 260 nm for each temperature. The temperature range was from 40 to 98°C with a 1°C increase at 2 min intervals. The results were automatically tabulated as change in absorbance at 260nm per change in temperature $(\Delta A/\Delta T)$ at each interval. The temperature at which the maximum change was recorded was taken as the T_M value.

The absorbance at 260 nm of all DNA samples was adjusted to a value of approximately 0.7 using the saline-citrate buffer, pH 7.0 (Appendix F).

A standard DNA sample of *Escherichia coli* (Calbiochem, Behring

Diagnosics Calif.) was dissolved in saline-citrate buffer and dialyzed against the same buffer overnight at 5°C. The *E. coli* DNA was included as a reference for each run and was used to determine the mole percent G+C values for each of the DNA samples by the following equation:

$$\text{mole\% G+C} = \text{mole \%G+C ref} + 1.99[T_M(x) - T_M(\text{ref.})]$$

where, x was the sample DNA and ref was the *E. coli* DNA mole% G+C (ref=50).

2.2.11. Immunological studies

Antiserum preparation

Antiserum to the purified T16 protease was prepared in random-bred New Zealand white rabbits. Four 100 µg injections of the purified enzyme in 1.0 ml of complete Freund adjuvant (Difco Laboratories, Detroit, Mich.) were given subcutaneously at 2 week intervals. The animals were sacrificed by heart puncture and the blood collected in sterile 30-ml tubes. The blood was stored at 5°C and allowed to clot. The clot was cut into quarters using a spatula and the serum gently decanted into a centrifuge tube. The serum was centrifuged (1,000 x g) for 20 min at 4°C. The supernatant obtained was the source of the antiserum. The gamma immunoglobulin (IgG) from the rabbit serum was prepared by sodium sulphate precipitation followed by DEAE- cellulose chromatography as described in Appendix G.

Immunoelectrophoresis

The immunoelectrophoresis of the samples of purified and crude T16 protease was performed according to the method of Scheidegger (1955) using Tris-barbitol buffer, pH 8.6. The immunoelectrophoresis gels were prepared as described in Appendix H. Approximately 10 μ g of the protease was put in each of the antigen wells and electrophoresis was carried out using an immunoelectrophoresis chamber (Gelman Sciences, Inc., Ann Arbor, Mich.) at 3 mA per slide constant current for 45 min using a Buchler 3-1500 power supply. The agar in the center trough was removed and filled with the anti-T16 protease IgG (0.8 mg in 200 μ l). The gel was incubated for 24 h at 5°C in a humid atmosphere to allow precipitin arcs to form.

Immunodiffusion tests

The antigenic relatedness of the proteases from several isolates was examined by the Ouchterlony double-diffusion test as described by Stollar and Levine (1963). The concentration of the IgG fraction of the rabbit antiserum was 4 mg/ml as determined by Lowry's protein determination method. The concentration of the antigens (crude enzyme extracts) which gave the sharpest precipitin band was determined by changing the antigen concentration. To allow the precipitin bands to form, the Ouchterlony slides (Appendix H) were incubated in a humid atmosphere at 5°C for 24 h or more.

Inhibition of protease activity by anti-T16 protease IgG

To evaluate the ability of the anti-T16 IgG to inhibit protease activity,

various concentrations of the IgG (1.2 and 2.4 mg per assay mixture) were preincubated with crude enzyme extracts for 20 min at 5°C. Equivalent amounts of IgG from an unimmunized rabbit were preincubated in the reaction mixture of the controls. The inhibition of protease activity under standard assay conditions was expressed as percentage of the activity in the control assays. The crude enzyme extracts were diluted to give uninhibited reaction rates of 0.5 enzyme units per assay mixture.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) methods are based on the use of antigens or antibodies that are linked to an insoluble carrier surface. The relevant antigen or antibody in a test solution binds and the complex is detected by an enzyme labelled antibody or antigen.

The method used for the detection of antigen (T16 protease) by the double-antibody technique has been described by Voller *et al.* (1976).

The wells of the polystyrene culture plate (Benton Dickinson and Co., Oxnard, Ca.) were sensitized with 0.3 ml of the anti-T16 protease IgG fraction (1.2mg) overnight at 4°C. The wells were washed with phosphate-buffered saline containing Tween 20 (PBS-Tween) from a wash bottle, and gently agitated for 3 min. This procedure was repeated three times and after the final wash the plates were shaken dry. Next, various concentrations of purified T16 protease (120-840 µg) made up to 0.3 ml with PBS-Tween, were added, followed by a 2 h incubation

period at room temperature. The wells were again washed with PBS-Tween as before. Horseradish peroxidase labelled anti-T16 IgG (0.3 ml) was then added to each well. The conjugation of horseradish peroxidase (HRPO) to anti-T16 protease IgG was carried out according to the method of Nakane and Kawaoi (1974) as described in Appendix I. The plate was incubated for 3 h at room temperature. The wells were again washed. The horseradish peroxidase enzyme was detected by adding 0.3 ml of the aminosalicylic acid and hydrogen peroxide substrate. The reaction was stopped after 1 h by the addition of 0.05 ml of 3 N NaOH. The total volume was made up to 1.0 ml with deionized water. The absorbance of the contents of each well was read at 449 nm using the model UV-260 spectrophotometer. The control well was treated the same way as the sample well except 0.3 ml of 1% bovine serum albumin was substituted for the T16 protease.

*HRPO substrate preparation: 8 mg of 5-aminosalicylic acid (Sigma) was dissolved in 10 ml of warm deionized water. The solution was cooled and stored at 4°C. Immediately before use, the solution was warmed to room temperature and 1 N NaOH was added to give pH 6.0.

A 1 ml solution of hydrogen peroxide (0.05% in deionized water) and 10 ml of the aminosalicylic acid solution were mixed. This was the final substrate and was used immediately.

Chapter 3

RESULTS

3.1. Protease purification and properties

Purification

The affinity chromatography of the crude, partially purified T16 extract using CBZ-DL-phenylalanine TETA Sepharose 4B column material, resulted in two major peaks (Fig. 3-1). The first peak consisted of the nonabsorbed material, with no protease activity associated with it. The second peak, released by the elution buffer, contained most of the protease activity. A summary of the purification procedure is shown in Table 3-1. A 150-fold increase in purity, with a 51% recovery was achieved. However, a significant decrease was noted following the ultrafiltration step. Similar profiles were obtained for the other five proteases with recoveries of 38-59% before concentration by ultrafiltration (Table 3-2). The loss of active enzyme and protein during the concentration step may be a result of autodigestion or absorption of the protein to the filter.

Polyacrylamide gel electrophoresis, with 7.5% gels at pH 9.5, of each of the affinity purified enzymes resulted in a single protein band upon staining with Coomassie Brilliant Blue. Figure 3-2 shows the PAGE gel of T16. The analytical

Figure 3-1: Protease purification by affinity chromatography

Concentrated crude T16 protease extracts dissolved in the start buffer (25 mM sodium acetate pH 5.8 containing 100 mM NaCl and 10 mM CaCl_2) were applied to a column of CBZ-DL-phenylalanine TETA Sepharose 4B. The bound protein was eluted with eluting buffer, 100 mM Tris-HCl pH 8.0 containing 0.5 M NaCl and 10 mM CaCl_2 . Fractions (approximately 3 ml) were collected and the protein in them was monitored at 280 nm. Protease activity was determined by the standard protease assay method.

Figure 3-1: Protease purification by affinity chromatography

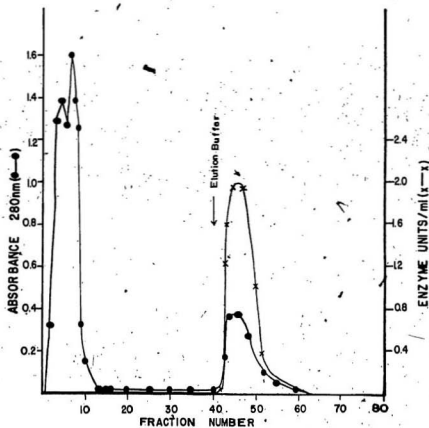


Table 3-1: Purification of protease from *P. fluorescens* T16

Step	Vol (ml)	Total Protein(mg)	Enzyme ^a Units	Specific ^b Activity	Degree of Purification	% Recovery
Crude extract	550	2760	69	0.025	1	100
Concentrated by lyophilization	50	163	63	0.385	15	91
Affinity chromatography	27	6.2	35	4.5	180	51
Concentrated by ultrafiltration	3	4.8	15	3.43	137	22

a. One enzyme unit is the amount of enzyme required to produce 1 μ mole of tyrosine equivalent per min under standard assay conditions.

b. Enzyme units per milligram of protein.

Table 3-2: Degree of purification and recoveries of psychrotroph protease by affinity chromatography^a

Protease	Specific ^b Activity	Degree Purification (fold)	Recovery ^c %
T6	2.3	163	47
T13	3.9	140	42
T20	4.8	200	59
T22	3.8	167	51
T26	4.0	158	38

^a Using CEZ-DL-phenylalanine TETA Sepharose 4B.

^b Enzyme units per mg protein as described in Materials and Methods.

^c Percent recovery of purified enzymes before concentration by ultrafiltration.

Figure 3-2: Polyacrylamide gel
electrophoresis of T16 protease

The affinity chromatography purified T16 protease (40 μ g/gel) was electrophoresed on 7.5% polyacrylamide gels at a current of 2 mA per gel. The gels were fixed in 12% TCA for 1 h and then stained using 0.25% Coomassie Brilliant Blue R250 stain.

O-origin

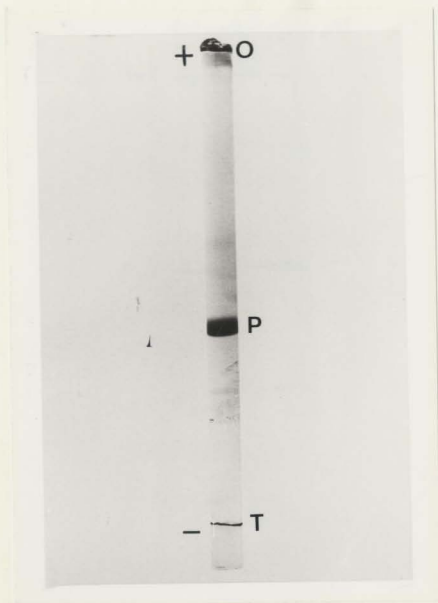
P-protease

T-tracking dye

+positive electrode

--negative electrode.

Figure 3-2: Polyacrylamide gel
electrophoresis of T16 protease



ultracentrifugation of the purified T16 enzyme showed a single symmetrical peak with a sedimentation coefficient of 3.9 S (Fig.3-3). These results indicate that the proteases obtained by affinity chromatography were homogeneous proteins.

3.2. Effect of assay conditions on protease activity

Effect of pH on protease activity

The influence of pH on the hydrolysis of soluble casein at 25°C by the psychrotroph proteases is illustrated in Figure 3-4. The protease activity was measured using 100 mM Tris-HCl buffer for the values between 7.0 to 9.0, while 100 mM citrate-phosphate buffer was used for pH 5.0 to 7.0. The pH profile for each of the proteases was similar, with maximum enzyme activity between pH 7.0 to 8.0. The enzymes retained some activity even at pH 5.0.

Effect of temperature on protease activity

The optimum temperature for protease activity at pH 7.5 for the six proteases ranged from 30° to 40°C, with a dramatic decrease in activity at 45°C (Fig 3-5).

The activation energies for the protease catalyzed casein hydrolysis between 5°C and 35°C were determined with the Arrhenius equation which relates reaction velocity to absolute temperature (Table 3-3). The observed activation energy for each protease was determined by multiplying the negative slope by a correlation coefficient of 2.303 R.

Figure 3-3: Ultracentrifugation of T16 protease

The purified T16 protease (1.5 mg/ml) was centrifuged at 60,000 rpm for 60 min in a Spinco Model E ultracentrifuge at 20°C. The sedimentation velocity was obtained from the Schlieren patterns.

Figure 3-3: Ultracentrifugation of T16 protease



Figure 3-4: Effect of pH on protease activity

The assay mixture contained in a total volume of 2.0 ml: crude extract, 0.2 ml; 0.5 ml substrate (1% soluble casein, adjusted to appropriate pH); and 1.3 ml of buffer, 100 mM Tris-HCl (pH 7.0-9.0) or 100 mM citrate phosphate (pH 5.0-7.0). Enzyme activity expressed as enzyme units per ml. One enzyme unit is the amount of enzyme extract that releases 1 μ mole of tyrosine equivalence per min per ml. The results are an average of three determinations.

Figure 3-4: Effect of pH on Protease Activity

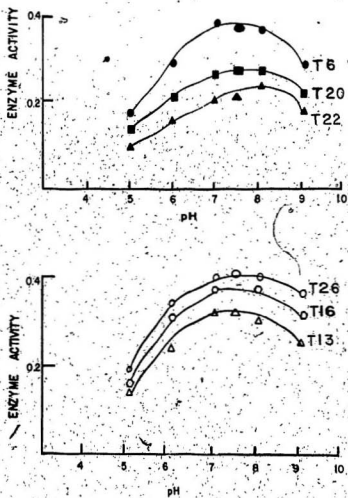


Figure 3-5: Effect of temperature on protease activity

Crude enzyme extracts were assayed by the standard protease assay method at various incubation temperatures (0 to 45°C) using 1% soluble casein as the substrate. Enzyme activity is expressed in enzyme units per ml.

T6 □ --- □

T13 ○ --- ○

T18 ■ --- ■

T20 ◇ --- ◇

T22 □ --- □

T26 ○ --- ○

Figure 3-5: Effect of temperature on protease activity

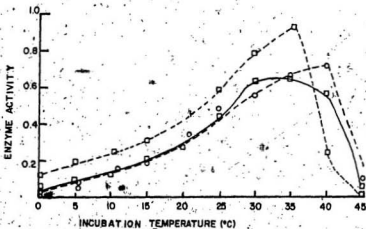
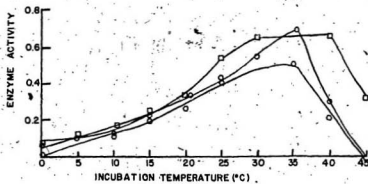


Table 3-3: Activation energies for protease-catalyzed casein hydrolysis^a

Protease	Activation Energy (J/mol)
T6	9,890 ± 1010
T13	13,900 ± 1130
T16	12,800 ± 780
T20	7,720 ± 940
T22	9,680 ± 1470
T26	9,860 ± 990

Effect of substrate concentration and incubation time

The optimal concentration of soluble casein for the activity of the T16 protease was approximately 2.5 to 5 mg/ml, with little change up to 10 mg/ml (soluble casein). The protease activity tended to decrease slightly with increased substrate concentrations in excess of 10 mg/ml (Fig. 3-6).

The hydrolysis of 1% soluble casein at 25°C as determined by the Hull's modified method protease assay, was found to be linear up to 40 min (change in absorbance of 0.85) followed by a gradual decline (Fig. 3-7). The decrease may have been a result of limited substrate and/or end product inhibition.

Protease activity using the pH stat method

The hydrolysis of soluble casein by the T16 protease could be measured using the pH stat method. The results were plotted as μ moles H^+ released per minute versus enzyme concentration at pH 8.0 (Fig. 3-8). The relationship was linear up to 90 μ g, with 40-50 μ g per assay being the lower concentration detected under the assay conditions described in Materials and Methods. The pH stat method was at least as sensitive as the modified Hull's method, which was included for comparison studies.

Figure 3-6: Effect of casein concentration on protease activity

The T16 protease (partially purified) activity with final concentrations of soluble casein (0-30 mg/ml) was determined using the standard protease method. The reaction mixture consisted of 0.2 ml enzyme, 1.0 ml substrate (5.0-60 mg) and 0.8 ml of 100 mM Tris HCl (pH 7.5). The enzyme activity was expressed as the change in absorbance at 280 nm. Bars indicate the standard error from the means (N=4).

Figure 3-6: Effect of casein concentration on protease activity

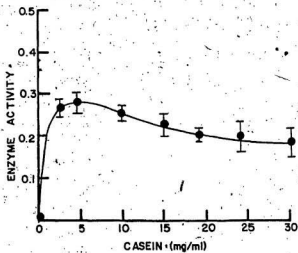


Figure 3-7: Effect of incubation time on protease activity

Partially purified T16 protease samples (0.2 ml) were incubated in the presence of 0.5 ml of 1% soluble casein and 1.3 ml of 100 mM Tris-HCl (pH 7.5) for different time intervals (5-70 min). The reactions were stopped by the addition of 1.0 ml of 5% TCA. The enzyme activity was expressed as the change in absorbance at 280 nm. The values are an average of three determinations. Bars indicate standard error of the mean.

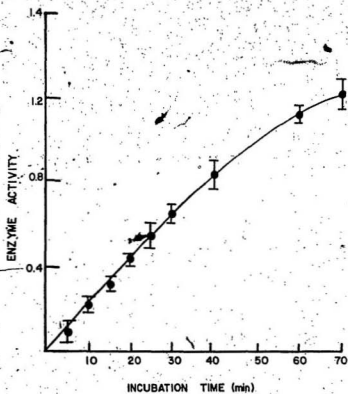
Figure 3-7: Effect of incubation time on protease activity

Figure 3-8: Determination of protease activity using the pH Stat method

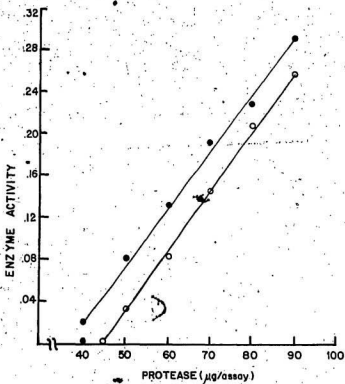
The hydrolysis of 2% soluble casein by the purified T16 protease was determined using an automatic pH-Stat/end point titrator as described in the Materials and Methods. The reaction mixture consisted of 5ml of 2% soluble casein in deionized water adjusted to pH 8.0. Different concentrations of enzyme were used (40-100 μg in 100 μl) and the activity was expressed as $\mu\text{moles H}^+$ released per mg enzyme per min.

The same enzyme concentrations were used for the modified Hull's method (standard assay), which was included for comparison to the pH stat assay method. Enzyme activity was expressed as the $\mu\text{moles tyrosine released per mg enzyme per min}$ at 25°C.

(●) Ph Stat method (○) Modified Hull's method

In each case the standard deviation from the mean of each point was <0.009 ($N=4$). The Least Squares lines were determined using the linear regression equation (Robbins and Van Ryzin, 1975). The correlation coefficients (r) are > 0.99 .

Figure 3-8: Determination of protease activity using the pH Stat method



3.3. Effect of protease inhibitors and activators

Metal ions and-protease inhibitors

At concentrations of 2 and 4 μ moles per assay mixture (1 and 2 mM) the metal ions Hg^{2+} , Zn^{2+} , Fe^{2+} , Cu^{2+} and Co^{2+} caused significant inhibition of the T16 protease activity. There was little effect on the protease activity by Ca^{2+} , Mg^{2+} and Mn^{2+} (Table 3-4).

The results of the atomic absorption spectral analysis of the purified proteases T13, T16 and T20, for the presence of Ca^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} are presented in Table 3-5. Only Ca^{2+} and Mg^{2+} were found to be present in significantly detectable amounts, although Zn^{2+} was only determined for one protease (T13) and found to be present at a concentration equivalent to Mg^{2+} .

The sensitivity of the T16 protease to a number of protease inhibitors is shown in Table 3-6. Significant inhibition was caused by the metal chelating agents (*o*-phenanthroline, EDTA, 8-hydroxyquinoline and 2,2-bipyridyl). Inhibition was also noted with *p*-chloromercuribenzoate, which suggests that there is cysteine present in the active site of the protease.

Reactivation of apoenzymes by metal ions

An inactive form of the proteases (apoenzyme) was obtained by dialyzing the enzymes against 100 mM EDTA in 20 mM Tris-HCl (pH 7.5). Different degrees of reactivation of the apoenzymes were achieved in the presence of added

Table 3-4: Effect of metal ions on protease (T16) activity

Metal Ions	Conc. (μ mol/assay)	Percent Activity
CaCl_2	2	105 ± 11
	4	107 ± 9
MnCl_2	2	102 ± 16
	4	107 ± 15
MgCl_2	2	109 ± 7
	4	110 ± 20
CoCl_2	2	77 ± 7
	4	69 ± 11
CuSO_4	2	38 ± 4
	4	16 ± 4
ZnSO_4	2	37 ± 8
	4	44 ± 12
FeCl_2	2	72 ± 21
	4	44 ± 13
HgCl_2	2	43 ± 5
	4	28 ± 9

Metal ions preincubated for 20 min at 5°C with enzyme in Tris-HCl buffer (pH 7.5). Activity of enzyme with no added metal ions was taken as 100%, using the standard protease assay method.

The values are an average of three determinations.

Table 3-5: Metal ion content of proteases

Protease	Metal ions (g atoms/mole)			
	Ca	Mg	Mn	Zn
T13	5.0	0.3	<0.1	0.5
T16	8.2	2.82	<0.1	nd
T20	8.0	4.0	<0.1	nd

The metal ion content of the purified proteases (0.3 mg protein/ml) were determined by atomic absorption spectrometry.

nd=not determined

Values obtained were from a single determination.

Table 3-6: Effect of protease inhibitors on protease T16

Inhibitor (μ mole/assay)	Concentration	Percent Activity
p-Chloromercuribenzoate	1.0	98 \pm 7
	2.0	42 \pm 11
PMSF ^a	2.0	100
	5.0	95 \pm 2
α -phenanthroline	1.0	0
	2.0	0
EDTA ^b	1.0	17 \pm 8
	2.0	8 \pm 8
8-Hydroxyquinoline	2.0	50 \pm 5
	4.0	25 \pm 9
2,2'-Bipyridyl	1.0	83 \pm 2
	2.5	66 \pm 7
Pepstatin	2.5	100
	5.0	100
Antipain	2.5	98 \pm 1
	5.0	84 \pm 4
Soybean trypsin inhibitor	1.0	100
	10	100
Cysteine	1.0	100
	2.0	100
N-Ethylmaleimide	1	100
	2	100

Inhibitors were preincubated with the enzyme in buffer (Tris-HCl, pH 7.5) for 20 min at 5°C. The activity was determined by the standard protease method. The percent activity was determined in relation to a control assay without the inhibitors. The values are an average of three determinations.

^a PMSF = phenylmethylsulfonylfluoride

^b EDTA = ethylenediamine tetraacetic acid

Table 3-7: Reactivation of EDTA-treated proteases with metal ions

Metal ions	Concentration (μ mol/assay)	T6	% Relative activity				
			T13	T18	T22	T26	T20
CaCl ₂	0	0	0	0	0	0	0
	4	25	24	92	34	32	87
	6	32	22	90	37	28	nd
MnCl ₂	0	0	0	0	0	0	0
	4	11	7	77	5	8	61
	6	9	4	88	0	4	100
MgCl ₂	0	0	0	0	0	0	0
	4	12	5	83	2	5	100
	6	9	4	88	0	4	100
ZnSO ₄	0	0	0	0	0	0	0
	4	0	0	0	0	0	0
	6	0	0	0	0	0	0

The reactivation of EDTA - treated protease was expressed as a percentage of the activity of the non EDTA - treated protease.

The enzyme activity was determined using the standard protease assay method. The values are an average of three determinations.

The apoenzymes were obtained by dialyzing the protease against 100 mM EDTA Tris-HCl (pH 7.5)

nd = not determined

Ca^{2+} , Mg^{2+} and Mn^{2+} (Table 3-7). Reactivation was greatest for proteases T16 and T20. No restoration of enzyme activity was detected in the presence of added Zn^{2+} .

3.4. Thermal stability studies

Heat resistance of the T16 protease

The partially purified enzyme sample (T16) was heated at 50, 90, 120 and 150°C for up to 10 min in 100 mM Tris-HCl, pH 7.5. The greatest decrease in protease activity occurred at 150°C, followed by 50, 120 and 90°C (Fig. 3-9), although significant activity remained (18%) following exposure at 150°C for two minutes. For each temperature, except 150°C, the rate of activity loss appeared to be biphasic, with a rapid decrease during the first two minutes, followed by a more gradual decline with increased exposure time.

The D-values for the protease at 50, 90, 120 and 150°C, estimated from the plot of log % activity remaining vs exposure time (Fig. 3-10), were 6.0, 26.7, 7.2 and 2.2 min, respectively.

Effect of metal ions and lactose on heat stability

The presence of either Ca^{2+} or Mg^{2+} during the heat treatment of the T16 protease (90°C for 10 min) had the effect of increasing its heat stability. The protease activity was approximately three times greater for the heat-treated protease which had the added metal ions present than the sample without added Ca^{2+} or Mg^{2+} (Table 3-8).

Figure 3-9: Heat stability of the T16 protease

The T16 protease (0.6ml) was placed in heat sealed glass ampoules and subjected to temperatures of 50°C and 90°C in a water bath, and 120°C and 150°C in a controlled oil bath. After different lengths of exposure time (0-10 min) the enzyme was removed, centrifuged and the supernatant assayed for protease activity using the standard assay method. Bars indicate the standard error from the means (N=4).

50°C (●); 90°C (◐); 120°C (◑); 150°C (◒)

Figure 3-9: Heat stability of the T16 protease

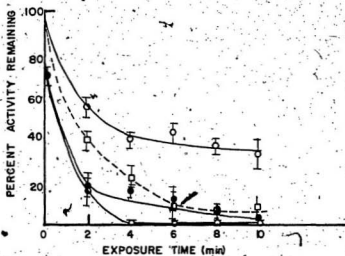


Figure 3-10: Determination of rate of activity loss during heat treatment

Plot of the log of residual activity of the T16 protease after heat treatment (as described in the previous figure) versus time. Only those residual activity values after the rapid decrease in protease activity were used. The D-values (time required for 90% inactivation) were determined for 50, 90, 120 and 150°C. The Least Squares lines were drawn using the linear regression equation. The correlation coefficient for each line is > 0.90 .

Figure 3-10: Determination of rate of activity loss during heat treatment

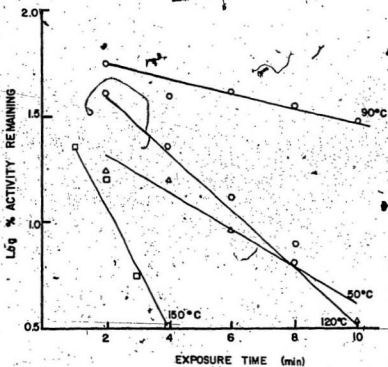


Table 3-8: Effect of Ca^{2+} and Mg^{2+} on the heat-stability of T16

Unheated Control	% Relative Residual Activity 100
Heat-treated: $\mu\text{moles CaCl}_2/\text{ml}$	
0	16 ± 4
5	29 ± 7
10	55 ± 13
20	49 ± 10
40	44 ± 9
Heat-treated: $\mu\text{moles MgCl}_2/\text{ml}$	
0	16 ± 3
5	55 ± 5
10	49 ± 8
20	49 ± 5
40	54 ± 11

Enzyme sample was heated at 90°C for 10 min in the absence and presence of varying amounts of metal ions.

The protease activity was determined soon after cooling on ice using the standard protease method. The values are an average of 4 determinations.

Table 3-9: Effect of the addition of metal ions on heat-treated T16 protease.

Unheated Control	% Relative Residual Activity ^a
	100
Heat-Treated: μmoles CaCl ₂ /ml added following heat-treatment	
0	22 ± 3
10	29 ± 5
20	26 ± 3
μmoles MgCl ₂ /ml added following heat-treatment	
0	21 ± 4
10	21 ± 6
20	21 ± 3

^a The metal ions were added to the enzyme sample following heat-treatment at 90°C for 10 min and preincubated for 1 h at 5°C before assaying for protease activity using the standard assay method. The values are an average of 4 determinations.

Table 3-9 shows the results of the effect of the addition of metal ions following the heat treatment of the T16 protease. Both Ca^{2+} and Mg^{2+} were found to be ineffective at reversing the inactivation of the protease due to the heat treatment. Lactose did not appear to have any effect on the heat stability of the protease (Table 3-10).

When stored overnight on ice the activities of the heat-treated proteases were found to be significantly higher than their activities soon after heating. This reactivation phenomenon after cold storage was most dramatic for the T13 protease, with an increase from 4% of the residual activity immediately following heat treatment to 26% after 24 h in ice (Table 3-11).

3.5. Physicochemical properties

Amino acid analysis

The amino acid composition of six of the affinity chromatography purified psychrotrophic proteases are listed in Table 3-12. For all proteases the predominant amino acids were aspartic acid, serine, glycine and alanine. Proteases T13 and T26 differed from the others with relatively low concentrations of glutamic acid.

The molecular weights estimated from the amino acid residues of the proteases ranged from 39,000 to 42,000 and are in agreement with the values obtained by gel filtration.

The N-terminal amino acid for three of the proteases (T13, T16, and T20)

Table 3-10: Effect of lactose on the heat-stability of T16 protease

Unheated Control	% Relative Residual Activity 100
Heat Treated/ mg Lactose/assay	
0	21 ± 1
0.5	20 ± 4
1.0	23 ± 6
2.5	21 ± 3
5.0	23 ± 4

Enzyme sample was heated at 90°C for 10 min in the presence and absence of varying amounts of lactose. The protease activity was determined soon after cooling in ice, using the modified Hull's method. The reaction mixture consisted of 0.2 ml of partially purified T16 protease, 0.5 ml soluble casein (1%) and various concentrations of lactose (0-5 mg) in 1.3 ml of 100 mM Tris-HCl (pH 7.5). The values are an average of 3 determinations.

Table 3-11: Reactivation of heat-inactivated proteases

Protease	<u>% Relative Activity</u>		After Cold Storage ^b
	Before Heating	After Heating ^a	
T13	100	4	26
T16	100	19	48
T20	100	34	89

^a Enzyme samples (partially purified) were heated at 90°C for 10 min in sealed glass vials. Protease activity was determined soon after cooling in ice using the standard protease assay method.

^b The heat-treated samples were left in ice (0-4°C) for 24 h before determining the protease activity.

The results are an average of two separate determinations.

Table 3-12: Amino acid composition of psychrotroph proteases

Amino acid	% Total					
	T6	T13	T16	T20	T22	T 26
Asp	14.0	16.7	13.4	12.3	17.0	17.7
Thr	10.0	9.5	7.6	7.0	6.5	7.7
Ser	11.6	8.5	11.2	11.3	9.8	10.5
Glu	12.9	4.9	10.0	12.0	8.8	7.2
Pro	2.9	1.8	2.9	3.8	2.6	2.6
Gly	14.2	14.9	14.1	13.8	15.0	13.9
Ala	8.2	10.3	8.8	8.5	9.3	8.7
Val	4.6	5.6	7.3	5.8	5.7	5.1
Met	0.8	0.7	0.7	0.5	1.0	0.5
Ile	2.9	3.6	3.7	3.5	3.6	3.9
Leu	5.3	7.5	6.3	6.8	6.5	6.7
Tyr	3.4	4.1	2.9	2.3	3.4	5.1
Phe	3.2	4.9	3.7	3.0	3.6	4.1
Lys	2.6	2.6	2.9	3.0	2.8	2.4
His	2.1	2.3	1.9	2.3	2.1	2.1
Arg	1.3	2.1	2.4	3.3	2.1	1.8
Trp ^a	-	0.7	0.4	1.0	-	-
Total	379	392	411	400	387	389
N-terminal ^b a.a	nd	thr	thr	thr	nd	nd
Mol.wt(kd) ^c	39	41	42	42	40	41
Hydro- phobicity ^d (J/mol)	2879	3611	3322	3197	3184	3422

Amino acid residues were detected following hydrolysis in 6 N HCl at 110°C for 24, 48 and 72 h.

^aTryptophan was determined following hydrolysis in 3 N mercaptoethanesulfonic acid (Penke *et al.*, 1974).

^bN-terminal amino acid determined by the dansyl chloride method (Wood and Wang, 1967).

^cEstimated from the molecular weight of the individual amino acid residues

^dBy the method of Bigelow (1967).

nd = not determined

was determined by the dansyl chloride method. In each case the N-terminal was threonine, as indicated by their relative mobilities (Rf) to standard amino acids during thin layer chromatography.

Molecular weight and subunit structure

The relative molecular weight of the purified T16 protease was determined by disc gel electrophoresis with 10% gels in the presence of 0.1% SDS and 0.1% 2-mercaptoethanol. A single band (Fig. 3-11) was obtained with an estimated molecular weight of 37,800 \pm 1,500 when compared to a set of standard proteins. On the basis of this result the protease appears to be a monomer with a single subunit.

Molecular weight determination by gel filtration

Crude concentrated enzyme samples were passed through a calibrated Sephadex G-150 column with 20 mM Tris-HCl (pH 7.5). The molecular sizes of the proteases were estimated by comparison to standard proteins of known molecular weights (Fig. 3-12) and are listed in Table 3-13. All of the samples had a single protease in the crude enzyme preparation, with molecular weights of 38,000 to 40,000 daltons. On the basis of these results the proteases appear to be monomers consisting of single subunits.

Metsger difference index

The difference index (DI) values for 15 pairs of proteases from psychrotrophs

Figure 3-11: SDS polyacrylamide gel electrophoresis of T16 protease

The purified T16 protease was analyzed by electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS). The protein (40-80 μ g) was reduced by boiling in 0.1% SDS and 0.1% 2-mercaptoethanol before application to the gels.

Gel 1- Purified T16 protease, P-protease, O-origin, T-tracking dye

Gel 2- Molecular weight standards: A-phosphorylase, B, B-bovine serum albumin, C-ovalbumin, D-carbonic anhydrase, E-soybean trypsin inhibitor, F-lysozyme, O-origin, T-tracking dye.

Figure 3-11: SDS polyacrylamide gel electrophoresis of T16 protease

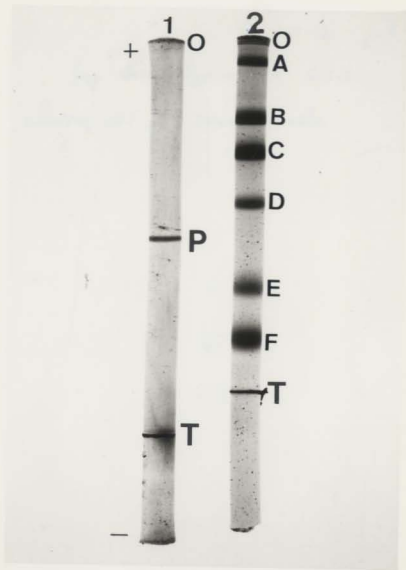


Figure 3-12: Gel filtration standard curve

Proteins of known molecular weights were passed through a Sephadex G-150 chromatography column. The column was washed with 20 mM Tris-HCl, pH 7.5 and the volume at which the protein was eluted (V_e) was noted. A standard curve was prepared by plotting K_{av} versus log molecular weight.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

V_o = void volume as determined with blue dextran (48 ml)

V_t = total bed volume of column (250 ml)

V_e = elution volume of protein

The standard proteins were, BSA (66,200), catalase (60,000), ovalbumin (45,000), trypsin (23,000), lysozyme (14,400) and cytochrome C (11,700).

Figure 3-12: Gel filtration standard curve

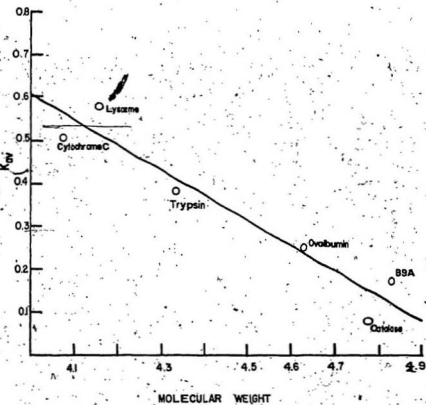


Table 3-13: Molecular weights of proteases by gel filtration

Protease	Molecular Weights
T6	39,800
T13	38,000
T16	38,000
T20	39,800
T22	39,800
T26	38,000

Molecular weights were determined by passing crude concentrated samples through a calibrated Sephadex G150 column. Protease activity in the fractions from the column was determined by the standard assay method described in Materials and Methods. The values obtained are an average of two determinations.

Table 3-14: Difference index

Protease						
	T6	T16	T22	T26	T20	T13
T6	0					
T16	7.2	0				
T22	9.7	6.1	0			
T26	10.5	7.8	5.4	0		
T20	7.4	4.9	8.7	10.2	0	
T13	13.1	11.7	7.4	7.1	14.5	0

^a The difference index values were calculated according to the method of Metzger *et al.* (1968) using the amino acid composition of each of the purified proteases listed in Table 3-4.

Isoelectric point (pI) determination

The pI values for each of the six proteases were estimated by chromatofocusing (Table 3-15). Crude enzyme extracts were applied to a polybuffer exchanger (PBE) column and eluted with a pH gradient using polybuffer 74 (Pharmacia). In each case a single protease peak was eluted from the column (Fig. 3-13).

Carbohydrate content

Analysis of the T16 protease for amino sugar content showed that both glucosamine and galactosamine were present (Table 3-16). This indicates that the protease can be considered to be a glycoprotein.

Hydrophobicity of the proteases

The average hydrophobicities of the proteases were estimated from their individual amino acid content using the method of Bigelow (1967). The values obtained were from 2879 to 3611 joules/mole (Table 3-12).

The relative hydrophobicities of three proteases were determined by hydrophobicity interaction chromatography (HIC) using Octyl-Sepharose 4B. Figure 3-14 represents the data obtained for protease T16. A summary of the results for all three proteases is presented in Table 3-17. The higher concentration of ethylene glycol (lower ammonium sulphate concentration) required to elute the T13 protease from the gel, as opposed to that needed for

Table 3-15: Isoelectric point (pI) estimation by chromatofocusing

Protease	pI
T8	5.6
T13	5.7
T16	5.7
T20	6.2
T22	5.5
T26	6.2

The pI was determined by chromatofocusing using a PBE column and Polybuffer-74, and is the average of two determinations.

Figure 3-13: Chromatofocusing of T16 protease

Crude T16 enzyme (10 mg) dialyzed against the start buffer (25 mM Imidazole-HCl, pH 7.4) was applied to a Polybuffer Exchanger column and eluted at its isoelectric point (pI) with polybuffer 74 diluted 1:8 with distilled water and adjusted to pH 4 with HCl. The pH () value of each fraction (3 ml) () the proteolytic activity determined using the standard protease assay; () absorbance at 280 nm.

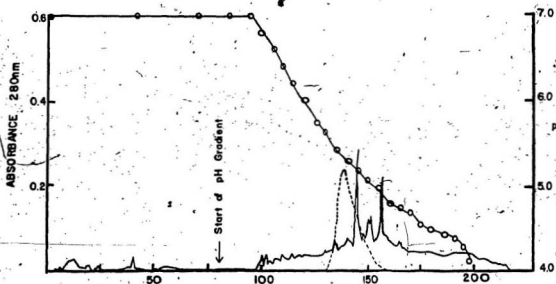
Figure 3-13: Chromatofocusing of T16 protease

Table 3-16: Carbohydrate content of T16 protease^a

Amino sugar	nmole/mg protein ^b
Glucosamine	31.1
Galactosamine	2.8

^a Amino sugar content of affinity chromatography purified T16 protease by the method described by Black *et al.* (1970).

^b Values obtained were from a single determination.

Figure 3-14: Hydrophobicity interaction chromatography

Sample: 10 ml (5 mg) of T16 crude enzyme extract in 100 mM sodium phosphate buffer pH 6.8 25% saturated with ammonium sulphate (start buffer). Sample was added to an Octyl-Sepharose CL-4B column, bed volume 8 ml. After sample addition, elution was continued with 200 ml of the start buffer, followed by a gradient of decreasing ammonium sulphate (AmSO_4) concentration and increasing ethylene glycol concentration (final concentrations of 0% and 25% respectively.) Enzyme activity () was determined by the standard protease assay; protein was monitored at 280 nm ().

Figure 3-14: Hydrophobicity interaction chromatography

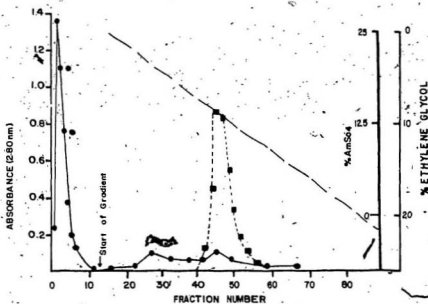


Table 3-17: Hydrophobicity interaction chromatography.

Elution from gel ^a			
Protein.	%Ethylene Glycol	% Ammonium Sulphate	HI (J/mole) ^b
T13	10.0	12.5	3611
T16	9.5	12.6	3322
T20	8.5	12.8	3197
catalase	10.3	12.4	5020
myoglobin	11.8	12.0	4561

^a The percentage of ethylene glycol and ammonium sulphate required to release the bound protein from the Octyl-Sepharose 4B column material. The values obtained are an average of two separate determinations.

^b HI = hydrophobicity values obtained from the amino acid content of each protein using the method of Bigelow (1967) were included for comparison.

be intermediate. These results agree with the relative hydrophobicities determined using Bigelow's method.

3.6. Substrate specificity of proteases

Specificity of proteases to protein substrates

The ability of the extracellular proteases to use a variety of proteins as substrates was determined using the ninhydrin protease assay method. The results in Table 3-18 show that, except for protease T16, α -casein was the preferred substrate, although significant hydrolysis of the other caseins was also evident. The other milk proteins, α -lactalbumin and β -lactoglobulin were not hydrolyzed. The non-milk proteins hemoglobin, bovine serum albumin, and ovalbumin were all broken down to various degrees by the different proteases.

Five of the purified proteases were examined to determine if they exhibited collagenase and elastase activity. All five of the enzymes were able to hydrolyze both collagen and elastin (Table 3-19).

Specificity of T16 protease to synthetic substrates

The specificity of the T16 protease to a number of synthetic peptides is shown in Table 3-20. The protease was able to hydrolyze both dipeptides and short polypeptides. The action of the T16 protease on the low molecular synthetic substrates BAPA and TAME was also examined. The protease was only able to hydrolyze BAPA, with a K_m of 0.59 mM (Fig. 3-15). The catalytic efficiency was considerably less than that of trypsin ($K_m = 0.003$ mM) for BAPA.

Table 3-18: Specificity to protein substrates

Substrate	% Relative Activity					
	T8	T13	T16	T20	T22	T26
Soluble casein	100	100	100	100	100	100
α -casein	142	100	56	108	118	130
β -casein	79	64	37	76	100	66
κ -casein	89	nd	165	97	66	105
Hb	54	39	96	18	44	47
BSA	41	57	54	6	21	58
Ovalbumin	14	19	42	11	21	5
α -Lactalbumin	0	0	0	0	0	0
β -Lactoglobulin	0	0	0	0	0	0
γ -casein	68	72	81	nd	nd	67

nd = not determined, Hb = hemoglobin, BSA = bovine serum albumin

The activity was expressed as a percentage of the activity obtained when soluble casein was used as the substrate for Hull's modified assay method. The values are an average of three determinations.

Table 3-19: Elastase and collagenase activity of proteases

Protéase	Activity (enzyme units/mg)		Caseinase ^c
	Collagenase ^a	Elastase ^b	
T13	0.478 ± 0.127	0.160 ± 0.032	31.2 ± 0.7
T16	0.622 ± 0.204	0.101 ± 0.016	31.2 ± 0.4
T20	0.785 ± 0.155	0.092 ± 0.029	32.4 ± 0.7
T22	0.835 ± 0.093	0.147 ± 0.011	34.2 ± 0.3
T26	0.835 ± 0.131	0.120 ± 0.015	33.6 ± 1.6

^a Collagenase activity was expressed as the μ moles of amino acids released per h per mg enzyme as described in Materials and Methods (N=4).

^b Elastase activity was defined as mg elastin solubilized per h per mg enzyme as described in Materials and Methods (N=4).

^c Caseinase activity was defined as the μ mole tyrosine released per h per mg enzyme (Hull, 1947) (N=4).

Table 320: Protease activity against synthetic peptides

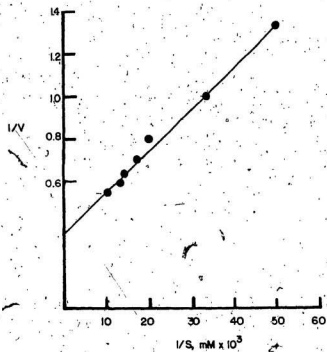
Synthetic Peptides (1 mM)	Specific Activity
N-CBZ-L-Tyrosyl-L-Ser	0
N-CBZ-L-Tyrosyl-L-Ala	$0.031 \pm .004$
N-CBZ-L-Tyrosyl-L-Thr	$0.036 \pm .009$
N-CBZ-L-Tyrosyl-L-Leu	$0.019 \pm .003$
N-CBZ-L-Tryptophyl-L-Ala	$0.026 \pm .001$
N-t-Boc-Ala-Pro-Tyr-Gly-NHMe	$0.033 \pm .009$
N-t-Boc-Ile-Pro-Tyr-Ala-NHMe	$0.039 \pm .007$
N-t-Boc-Leu-Pro-Tyr-Ala-NHMe	$0.035 \pm .007$
N-t-Boc-Ala-Pro-Tyr-Ala-NHMe	$0.017 \pm .006$
α -N-benzoyl-DL-arginine-p-nitroanilide	$0.180 \pm .012$

The degree of hydrolysis of peptides at 25°C by purified T16 protease (0.1 mg) was determined by the ninhydrin method (section 2.2.7.6) and is the average of three determinations.

Specific activity was expressed as μ moles of amino acid or p-nitroanilide released per h per mg enzyme protein.

Figure 3-15: Activity of T16 protease on L-BAPA substrate

The proteolytic activity of the purified T16 protease was measured using L-BAPA according to the method of Erlanger *et. al* (1961). The hydrolysis of different concentrations of substrate (0.1 to 2.0 mM) by the protease (50 μ g) was determined spectrophotometrically at 410 nm. The reaction velocity (V) was expressed as the change in absorbance per minute. The kinetic constant, K_m was determined from the double reciprocal plot. The Least Squares line was determined using the linear regression equation ($r=0.99$).

Figure 3-15: Activity of T16 protease on L-BAPA substrate

Effect of temperature on secondary structure of T16 protease

The circular dichroism (CD) spectra of the T16 protease at different temperatures were determined using a Jasco Spectropolarimeter J20-A (Fig. 3-16). The secondary structure was estimated based on the poly-lysine model of Greenfield and Fasman (1969). The T16 protease at 5 and 25°C was found to consist primarily of random coil (67%) and β -sheet (33%) with no detectable α -helix (Table 3-21). At temperatures in excess of 30°C the secondary structure of the protease tended to shift toward an increase of random coil with a subsequent decrease in β -sheet structure. The shift to the random or unordered form appeared to be greater at 45 to 55°C than at 95°C (Fig 3-16).

The thermal denaturation of a protein can be followed as a function of the decrease of ellipticity at 224 nm of the CD spectra obtained at different temperatures. The denaturation profile which was obtained for the T16 protease is shown in Fig. 3-17. Maximum unfolding of the protease occurred between 45 to 55°C, however at temperatures from 60 to 90°C the protease appeared to become more ordered, possibly due to refolding of the protein molecule.

3.7. Localization of active protease and precursor

The culture supernatant, the washed whole cells, the cell contents, and cell envelopes obtained by mechanical breakage of the whole cells were assayed for protease activity using soluble casein as the substrate. Protease activity was only detected in the culture supernatant (Table 3-22) indicating that only the extracellular form of the T16 protease is active. Osmotic shock treatment with $MgCl_2$ was used to selectively separate the T16 bacterial cells into

Figure 3-16: Circular dichroism (CD) spectra of the T16 protease

The circular dichroism spectra at various temperatures (25-95°C) of purified T16 (40 μ g) protease were obtained using a Jasco J-20 A Spectropolarimeter. The sample was scanned at wavelengths from 245 to 195 nm at a speed of 50 nm/min, with a sensitivity of 2 and a sampling time of 1 msec.

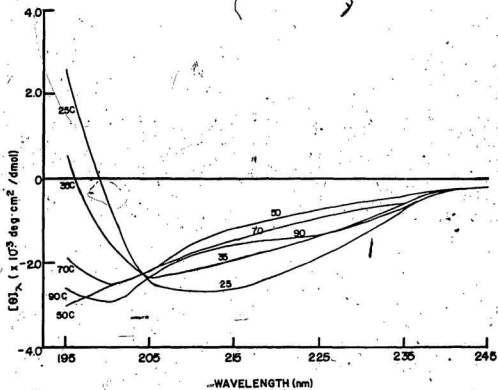
Figure 3-16: Circular dichroism (CD) spectra of the T16 protease

Table 3-21: Effect of temperature on secondary structure of protease T16

Temperature (°C)	% β Structure	% Random Coil
5	33	67
25	33	67
35	29	71
40	26	74
45	25	75
50	25	75
55	26	74
60	26	74
65	27	73
70	27	73
75	27	73
80	28	72
85	28	72
90	28	72
95	28	72

Based on the circular dichroism (CD) spectra recorded from 195-245 nm of the purified T16 protease (40 μ g) at 5-95°C. The secondary structure was calculated according to the poly-lysine-model of Greenfield and Fasman (1969). The values are the average of two separate determinations.

Figure 3-17: Thermal denaturation of the T16 protease

Approximately 100 μ l (40 μ g) of purified T16 protease in 20 mM Tris-HCl (pH 7.5 buffer) was heated in a water-jacketed sample cell, using a temperature regulated water bath, at 5°C increments (25 to 90°C). The sample was maintained at each temperature for 8 min. The extent of denaturation (unfolding) of the enzyme was determined by CD-measurements at 224 nm, with a Jasco J-20A Spectropolarimeter. The enzyme was assumed to be completely folded at 25°C.

Figure 3-17: Thermal denaturation of the T16 protease

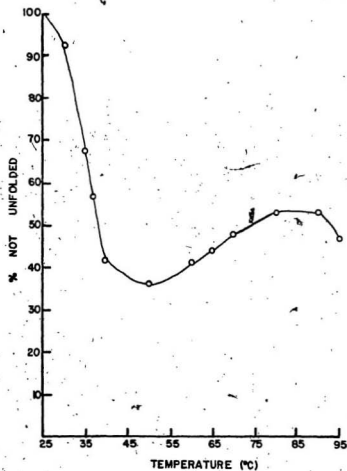


Table 3-22: Localization of active protease in cell fractions

Cell Fraction ^a	Protease Activity ^b (Enzyme units/ml)
Culture Supernatant	0.11
Washed Whole Cells	0
Cell Contents	0
Cell Envelopes	0

^aFractions obtained by mechanical breakage of cells as described in Materials and Methods.

^bProtease activity was determined using the standard assay method.

periplasmic and cytoplasmic fractions. The marker enzymes, alkaline phosphatase (periplasmic enzyme) and glucose-6-phosphate dehydrogenase (cytoplasmic enzyme) were assayed in each fraction to ensure that the osmotic shock treatment was successful. All of the alkaline phosphatase was associated with the $MgCl_2$ extract and $MgCl_2$ shock fluid, while glucose-6-phosphate dehydrogenase was only detected in the cell contents and cell envelopes, indicating that the osmotic shock treatment was effective (Table 3-23). A possible inactive form of the protease was detected in the $MgCl_2$ shock fluid (periplasmic) fraction by the formation of a precipitin band against the anti-T16 protease IgG. Figure 3-18 shows the immunodiffusion reaction for each of the fractions, as well as purified T16 protease, against the IgG. The precipitin band patterns between the active protease and the possible precursor is characteristic of antigenic non-identity.

Attempts to activate the precursor by selective cleavage using the active T16 protease and trypsin were not successful.

3.8. Mole percent G+C determination

Psychrotrophic bacteria from different geographical regions were compared based on the mole % G+C content of their DNAs. All of the 10 isolates were of milk origin and capable of producing heat-stable extracellular proteases. The mole % G+C content for each isolate was determined from the melting temperature (T_M) of their DNA as described in section 2.2.10. The values for all of the isolates were in the range of 58 to 62%, except for isolate O15 which had a mole % G+C content of 44 (Table 3-24). According to Palleroni (1984) the mole % G+C content of

Table 3-23: Localization of protease precursor

	Alkaline Phosphatase ^a (% of total)	Glucose-6-Phosphate Dehydrogenase ^b (% of total)
MgCl ₂ wash	15	0
MgCl ₂ Shock Fluid (Fraction II)	85	0
Cell Contents (Fraction III)	0	47
Cell Envelopes (Fraction IV)	0	53

^aAlkaline phosphatase activity was determined by the method of Garin and Levinthal (1960) using *p*-nitrophenylphosphate. *p*-Nitrophenol release was detected by absorbance at 420 nm.

^bGlucose-6-phosphate dehydrogenase activity was measured by the reduction of NADP⁺ as determined by change-in absorbance at 340 nm (Malamy and Horecker, 1964).

The activities were expressed as a percentage of the total detected for all fractions.

Figure 3-18: Localization of proprotease by the Ouchterlony double-diffusion test.

The double-diffusion test was carried out as described in Materials and Methods. The different fractions obtained from the osmotic shock treatment of the T16 cells (section 2.2.9.5) were put in the outer wells with the anti-T16 protease IgG in the center well. Purified T16 protease (10 μ g) was also included.

1-supernatant (Fraction I)

2-MgCl₂ wash

3-cell envelope (Fraction III)

4-cell contents(Fraction IV)

5-MgCl₂ shock fluid (Fraction II)

6-purified T16 protease

Figure 3-18: Localization of proprotease by the Ouchterlony double-diffusion test.



Table 3-24: Mole percent G + C determination

Isolate	Origin ^a	Mole % G+C
T6	N	60
T13	N	60
T10	N	60
T20	N	58
T22	N	60
T26	N	60
O13	O	59
O15	O	44
32A	O	58
33	O	60
49	O	58
240	A	62
M5	U	62
AFT7	I	60
AFT21	I	58
AFT36	L	60
2-2	B	60
2-1	B	62
22F	Ne	58

^aN=Newfoundland

O=Ontario

B=British Columbia

I=Ireland

A=Australia

U=U.S.A.

Ne= Netherlands

members of the genus *Pseudomonas* is between 58 and 70. A mole % G+C content of 44 is consistent with the genus *Altermonas* (Baumann *et al.*, 1972). Members of the genus *Altermonas* have properties that are similar to those of *Pseudomonas* except that their mole % G+C is different.

3.9. Immunological studies

Immuno-electrophoresis of protease T16

Samples of the purified and crude extracts of T16 were separated by electrophoresis as described in the Materials and Methods. Precipitin arcs developed against the anti-serum to the purified enzyme. A single precipitin arc was observed for both the crude and purified enzyme samples (Fig. 3-10) which indicates the presence of a single antigen.

Ouchterlony double-diffusion tests

All six of the proteases of the psychrotrophic pseudomonads, isolated from Newfoundland, produced strong precipitin bands against anti-T16 protease IgG with the qualitative immunodiffusion test (Fig. 3-20A). The lack of spur formation between these antigens in adjacent wells suggest that they share common antigenic determinants.

Crude enzyme extracts of the psychrotrophic bacteria from different geographical regions (Ontario, British Columbia, Ireland, U.S.A., Australia, and Netherlands) were also found to cross react with the antiserum to the T16 protease (Fig. 3-20B). Some proteases produced multiple precipitin bands, possibly indicating the presence of

Figure 3-19: Immunoelectrophoresis of the T16 protease

Approximately 10 μ l, (10 μ g), of the protease solutions were put into the wells on each side of the central trough. The proteins underwent electrophoresis for 45 min at 3 mA per slide. The agar in the center trough was removed and filled with the anti-T16 protease IgG (0.8 mg). The gels were incubated for 24 h at 5°C in a humid atmosphere to allow precipitation arcs to form.

A-purified T16 protease

B-crude T16 protease

Figure 3-19: Immunelectrophoresis of the T16 protease

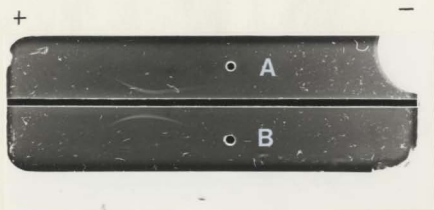


Figure 3-20: Ouchterlony double-diffusion of anti-T16 protease IgG and proteases from different geographical regions

The agar gels were prepared on microscope slides as described in Appendix H. The outer wells were filled with the different protease solutions (20 μ l of crude extract per well).

A. Proteases of isolates from Newfoundland:

- 1 T6
- 2 T13
- 3 T16
- 4 T20
- 5 T22
- 6 T26

B. Proteases of isolates from Ontario, British Columbia, Ireland, U.S.A., Australia, and Netherlands.

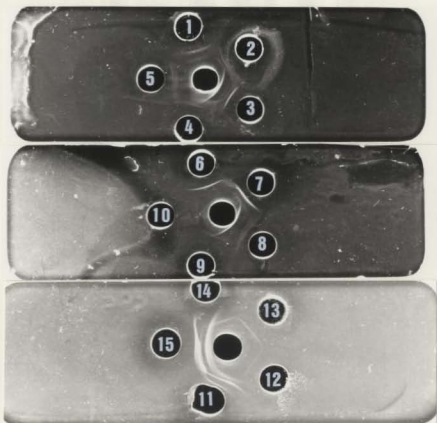
- | | | |
|-------------------------------------|-------|-----------|
| 1 BC 2-2 | 6 22F | 11 S63 |
| 2 BC 1-1 | 7 24O | 12 AFT7 |
| 3 M-5 | 8 015 | 13 AFT 21 |
| 4 O-13 | 9 32A | 14 AFT 36 |
| 5 O-40 | 10 33 | |
| 15 <i>P. fluorescens</i> ATCC 15456 | | |

Figure 3-20: Ouchterlony double-diffusion of anti-T16 protease IgG and proteases from different geographical regions

A



B



more than one protease or the formation of polymers during the long incubation period (4 days) required to allow the weak bands to become more intense.

Inhibition of protease activity by anti-T16 protease IgG

Antiserum prepared against purified T16 protease inhibited the protease activity in partially purified extracts of T16 as shown in Figure 3-21. Complete inhibition of activity was obtained at an IgG concentration of 3.8 mg, however, the degree of inhibition depends on the specific activity and concentration of the enzyme in the reaction mixture.

The inhibitory effect of the IgG on the T16 protease catalyzed hydrolysis of α -casein is shown in Figure 3-22. The double reciprocal plots which were obtained (Fig. 3-22) are characteristic of non-competitive inhibition. The apparent K_m 's were 0.03 mM while the V_{max} decreased from 2.01 to 1.54 M per min. in the presence of 0.5 mg of IgG (Table 3-26).

The effect of the IgG on the activity of the proteases from the other Newfoundland strains was also investigated (Table 3-27). To standardize the conditions the concentrations of the enzyme protein were adjusted such that the uninhibited reaction rates were approximately the same (0.5 enzyme units per ml). All of the proteases were inhibited to a similar extent by the IgG, with the T20 protease being the least sensitive.

Figure 3-21: Effect of anti T-16 protease IgG on protease activity

The activity of the purified T16 protease to α -casein was determined in the presence of different concentrations of anti-T16 protease IgG (0.5 mg/assay). The reaction mixture consisted of 100 μ l (40 μ g) enzyme, 0.5 ml of 2% soluble casein, IgG and 100 mM Tris-HCl buffer (pH 7.5) to a final volume of 2.0 ml. Activity was determined using the standard protease assay method. The inhibition was expressed as a percentage of the activity in the absence of IgG and is an average of three determinations.

Figure 3-21: Effect of anti T-16 protease IgG on protease activity.

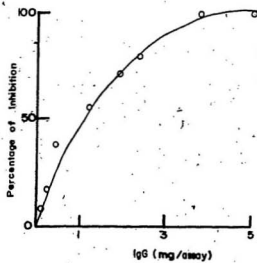


Figure 3-22: Effect of anti-T16 protease IgG on hydrolysis of α -casein

Double reciprocal plots obtained with different concentrations of α -casein as the substrate (S) and anti-T16 protease-IgG. The concentration of antibody per assay was 0 () and 0.5mg (). The T16 protease (0.5 enzyme units/ml) was incubated in the presence of the antibody for 20 min at 5°C before the addition of substrate. The assay mixture contained (final volume, 2 ml) 100 μ l enzyme, 0.5 mg IgG and 0.1 M Tris-HCl (pH 7.5) buffer. Enzyme activity was determined using the standard protease assay method.

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Figure 3-22: Effect of anti-T16 protease IgG on hydrolysis of α -casein

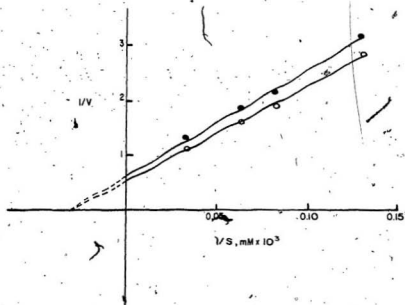


Table 3-25: Effect of anti-T16 protease IgG on hydrolysis of α -casein by T16 protease

anti-T16 IgG (mg)	K _m (mM)	V _{max} (M per min)
0	0.03	2.22
0.5	0.03	1.42

Apparent K_m and V_{max} values were determined from Lineweaver-Burke double reciprocal plots (Lineweaver and Burke, 1934).

Table 3-26: Inhibition of proteases by anti-T16 protease IgG

Protease	IgG(mg/assay)	% Inhibition
T6	1.2	38 \pm 7
	2.4	83 \pm 10
T13	1.2	42 \pm 4
	2.4	78 \pm 7
T16	1.2	56 \pm 11
	2.4	80 \pm 8
T20	1.2	54 \pm 6
	2.4	66 \pm 9
T22	1.2	80 \pm 14
	2.4	100 \pm 0
T26	1.2	65 \pm 16
	2.4	88 \pm 9

The crude enzyme extracts were diluted to give uninhibited reaction rates of 0.5 enzyme units per ml (N=3).

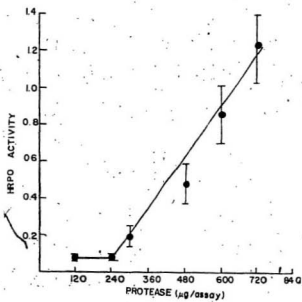
Enzyme linked immunosorbent assay (ELISA)

The protease assay using horseradish peroxidase linked anti-T16 protease IgG was effective at detecting the T16 enzyme. The lower detection limit of the ELISA method was approximately 300 μ g and appeared to be linear up to 720 μ g (Fig. 3-23). There was some variation with the actual values obtained upon repeating the assay as indicated by the standard deviations, particularly at the higher concentrations (600-720 μ g).

Figure 3-23: Enzyme linked immunosorbent assay (ELISA)

The protease assay using horseradish peroxidase linked anti-T16 protease IgG was effective at detecting the T16 enzyme. The wells of a polystyrene plate were coated with 0.3 ml anti-T16 protease IgG followed by various concentrations of purified T16 (120-840 μ g) in 0.3 ml. After washing, the bound T16 protease was detected by the addition of horseradish peroxidase conjugated anti-T16 protease IgG, as described in Materials and Methods. The relative amount of protease present in each well was determined by the horseradish peroxidase activity represented by change of absorbance at 449 nm. The values are an average of 4 determinations, the bars indicate standard error from the mean.

Figure 3-23: Enzyme linked immunosorbent assay (ELISA)



Chapter 4

Discussion

4.1. Purification

Psychrotrophic microorganisms are common contaminants of raw milk (Law, 1979; Cousin, 1982). Many psychrotrophs, particularly *Pseudomonas* species, are capable of producing heat-stable extracellular proteases which cause spoilage of milk and dairy products, especially UHT-sterilized products.

Most studies of these heat-stable proteases have used cell-free culture supernatants, while a few have used partially purified or purified enzymes. There have been only four other studies describing the purification of the extracellular heat-stable protease of pseudomonads of milk origin (Richardson, 1981; Stepaniak *et al.*, 1982; Fairbairn and Law, 1986b; and Mitchell *et al.*, 1986). The methods used by two of these groups consisted of ammonium sulphate precipitation, ion exchange chromatography with DEAE cellulose and gel filtration (Stepaniak *et al.*, 1982; and Mitchell *et al.*, 1986). The yields of purified enzyme were from 4 to 55%. Fairbairn and Law (1986b) included an additional step using chromatofocusing for the purification of the protease of *P. fluorescens* NCDO 2085 (AR11) with a final yield of 3.5% and a 158-fold increase in specific activity.

Richardson (1981) purified the protease of *P. fluorescens* B52 by ammonium sulphate precipitation and gel filtration on Sephadex G-75 and affinity chromatography using CBZ-D-phenylalanine TETA Sepharose-4B. The protease was purified 760-fold with a total yield of 40%.

In this study the proteases of six psychrotrophic pseudomonads were purified to homogeneity by affinity chromatography using CBZ-DL-phenylalanine TETA Sepharose-4B. In comparison to the other methods which have been used, this purification procedure resulted in a high degree of purity, with fewer steps. Because a major problem, when purifying proteases, is loss due to autodigestion, minimization of the number of purification steps should reduce the loss of active enzyme.

An initial attempt to purify the enzyme by affinity chromatography, using hemoglobin as the ligand to the Sepharose-4B support, was not successful. A brown pigment was associated with the fractions containing the active protease. This indicated that the bound enzyme may have degraded the hemoglobin ligand. This conclusion was supported by the observation that the affinity column material lost its ability to bind the protease after a single use.

Purity of the T16 protease preparation obtained by CBZ-DL-phenylalanine TETA Sepharose-4B affinity chromatography was indicated by disc gel electrophoresis and by the single symmetrical peak obtained with analytical ultracentrifugation. In addition, the specific activity of the fractions containing the active protease was essentially the same. The yield was 51% of the initial activity in the crude extract, with a 150-fold increase in purity.

Comparison of the properties of the proteases from this study with those reported for other proteases from psychrotrophs has shown some similarities and differences. The molecular weights of most psychrotroph proteases range from 40,000 to 49,000 (Barach *et al.*, 1976a; Stepaniak, Fox and Daley, 1982; Fairbairn and Law, 1986a; and Mitchell *et al.*, 1986). Mayerhofer *et al.*, (1973) isolated an extracellular protease of *P. fluorescens* P26 with a molecular weight of 23,000. The molecular weights of the proteases in this study as determined by gel filtration, SDS-PAGE, and amino acid content showed values of 38,000 to 42,000. This is in agreement with the previous studies. All proteases consisted of a single subunit. There have been no reports of psychrotroph proteases with multiple subunits.

The isoelectric point (pI) of a protein is the pH at which the overall charge of the exposed amino acid residues is zero, and is determined by its amino acid content and conformation. Proteins which are structurally closely related would be expected to have similar isoelectric points. The pI values of the six proteases in this study ranged from 5.5 to 6.2, indicating that there was very little variation of overall charge between these proteins. Fairbairn and Law (1986b) obtained a similar value, 5.4, for the AR11 protease, while in a study of six pseudomonad proteases by Mitchell *et al.* (1986) values from 5.1 to 8.25 were obtained.

Amino acid analysis of the six purified proteases showed that all had high levels of aspartic acid, glycine, serine and alanine with low methionine, histidine and arginine. The T13 protease stood out with its relatively low content of

glutamic acid. These results are in agreement with those of other studies on heat-stable proteases of pseudomonads. The B52 protease had high aspartic acid, serine, glycine and alanine levels (Richardson, 1981), while the proteases examined by Mitchell *et al.* (1986) were high in the low molecular weight residues, glycine and alanine. All appear to lack cysteine with the exception of the protease produced by *P. fluorescens* NCDO 2085 (AR11) which contained a low level of cysteine.

The Difference Index (Metzger *et al.*, 1968) assesses the homology of two proteins based on the differences in their content of each amino acid with a confidence coefficient of 0.95. DI values less than 10.0 indicated relatedness and values greater than 26.8 indicated unrelatedness. Those protein pairs with DI values in the range of 10.0 - 26.8 cannot be reliably identified as related or unrelated (Woodward, 1978). According to this criterion, 10 of the 15 pairs of proteins which were compared had DI values of less than 10 and could be considered to be closely related i.e. homologous (T6:T16, T6:T22, T6:T20, T16:T22, T16:T20, T16:T26, T22:T26, T22:T20, T22:T13 and T26:T13). Five pairs had intermediate values and could not be reliably called homologous or nonhomologous. None of the protein pairs could be considered nonhomologous. These results indicate that most, if not all, of the six proteases examined exhibited sequence homology and are derived from common ancestors.

4.2. Effect of temperature on protease activity

The influence of temperature on the relative activity of the six psychrotroph proteases was shown in Figure 3-5. The optimum temperature for the hydrolysis of soluble casein at pH 7.5 for each of the proteases was 35°C, with a rapid decline in activity as the incubation temperature was increased to 45°C. At 25°C between 80 and 100% of the maximum protease activity was retained. The significance of these findings to the dairy processor is obvious, because UHT-sterilized milk is usually stored without refrigeration for extended periods of time. Even heat-treated or raw milk held at refrigeration temperatures is potentially susceptible to these proteolytic enzymes because at a temperature of 5°C, 25 to 30% of the maximum protease activity still remained.

Alichandrais and Andrews (1977) obtained similar results with the protease of the psychrotroph, *P. fluorescens* AR 11. Its optimum activity was at 35°C with a retention of 72 and 33% activity at 20 and 4°C, respectively. The characterization of the proteases of six pseudomonads isolated from raw milk with respect to the effect of temperature gave comparable results with all being most active at 40°C while retaining considerable activity at 4 to 7°C (Gebre-Egziabher *et al.*, 1980a). The heat stable protease of *P. fluorescens* AFT 36, also maintained significant activity at 4°C (17%), however the optimum was higher, at 45°C (Stepaniak *et al.*, 1982).

The activation energies (E_a) for the hydrolysis of casein by the proteases ranged from 7,700 - 13,900 J/mol. These values are relatively low when compared

to mesophilic enzymes such as trypsin which has an E_a value of 50,220 J/mol. According to Somero and Low (1978) activation energies are generally proportional to adaptation temperature. This would suggest that these enzymes are adapted to function at low temperatures, resulting in relatively higher turnover numbers at low temperatures than for mesophilic or thermophilic enzymes.

4.3. Effect of pH on protease activity

The pH profiles for the proteases produced by each of the isolates are presented in Figure 3-4. The optimum pH for all of the proteases for the breakdown of casein was in the range of pH 7 to 8. At pH 6.5, the normal pH of milk, no less than 80% of the optimal protease activity was retained by each of the enzymes. Between 40 and 50% of their optimal activity was evident at pH 5, which corresponds to the pH of cultured dairy products. As a result, these proteases may play a role in the spoilage of such products. It was not possible to determine protease activity below pH 5, using casein, because the substrate tended to aggregate and come out of the solution.

Psychrotrophic pseudomonads, which produce extracellular proteases with similar pH optima, have been reported by other researchers. The protease of *P. fluorescens* B52 was active over a wide pH range (5.5 - 10.5) with optimal activity at pH 7. Maximal protease activity of proteolytic enzymes produced by a culture of *P. fluorescens* isolated from a frozen chicken pie was within the range of pH 7 to 8. However, the activity at pH 6.5 was relatively low, at



approximately 50% of the maximum (Peterson and Gunderson, 1960). The proteases of six pseudomonads, isolated from milk, had their optimal proteolytic activities between pH 6.5 and 6.8, with 65 to 75 percent of their optimal activities at pH 5.0 (Gebre-Egziabher *et al.*, 1980a).

4.4. Effect of inhibitors on protease activity

According to Hartley (1960) proteases can be classified into four groups based on their sensitivity to various protease inhibitors: serine, thiol, metal (or metal-chelator-sensitive), and acid proteases. Inhibition of the T16 protease was greatest in the presence of the metal chelating agents, *o*-phenanthroline, 8-hydroxyquinoline, 2,2'-bipyridyl and EDTA (Table 3-6). These results indicated that this protease is a metalloprotease. The T16 protease was also sensitive to *p*-chloromercuribenzoate (PCMB) which is characteristic of proteases with cysteine present in the active site. With respect to sensitivity to PCMB, this protease differs from all of the other psychrotroph proteases which have been characterized, with the exception of the protease of *P. fluorescens* AR11 (Fairbairn and Law, 1986b). However, unlike AR11, the T16 protease contains no detectable cysteine. This suggests that PCMB inhibited the protease activity by some other means besides binding to the sulfhydryl group of a cysteine residue. The T16 protease was found to be sensitive to heavy metal ions such as Hg^{2+} , Cu^{2+} and Zn^{2+} , thus the mercury component of PCMB may be responsible for the inhibition. This is further supported by the fact that the other thiol protease inhibitor, N-ethylmaleimide, did not inhibit the T16 protease.

All of the proteases were inhibited completely by 100 mM EDTA and reactivated by Ca^{2+} , Mg^{2+} and Mn^{2+} . The degree of reactivation varied depending upon the metal ion and the isolate. Complete restoration of the T20 apoenzyme activity was achieved by Mg^{2+} . The addition of Zn^{2+} had no effect on the EDTA-treated proteases. These results confirmed that these are metalloproteases with divalent cations (Ca^{2+} , Mn^{2+} or Mg^{2+}) being essential for proteolytic activity and a necessary part of the holoenzyme.

Most of the extracellular proteases of psychrotrophic pseudomonads have been classified as metalloproteases, however they tend to differ with respect to their metal ion requirements (Fairbairn and Law, 1986a). The protease of *Pseudomonas* B52 was reported to be activated by Co, Zn, Fe, Cu and Ca ions (Richardson, 1981), while *P. fluorescens* APT 36 produced a protease which could be activated following EDTA-treatment by Zn^{2+} , Ca^{2+} , Mg^{2+} and Co^{2+} (Stepaniak *et al.*, 1982). The proteases in this study were different from most other metalloproteases in that Zn^{2+} was not an effective activator of the apoenzymes.

Ca^{2+} was the predominant element present in each of the three purified proteases which were examined for their metal content (Table 3-5). The T16 and T20 proteases differed from T13 with their relatively high Ca^{2+} and Mg^{2+} contents. The difference was also reflected in the capacity of Mg^{2+} to reactivate these apoenzymes. Little, if any restoration of protease activity was noted for T13 which had 0.3 g atoms of Mg^{2+} per mole, while T16 and T20 apoenzymes

with 2.8 and 4.0 g atoms per mole respectively, were almost completely reactivated by added Mg^{2+} . The very low level of Mn^{2+} (less than 0.3 g atom per mole) in the proteases, which exhibited relatively high reactivations of their apoenzymes by Mn^{2+} (T13 and T16), suggests that Mn^{2+} can be substituted for either Ca^{2+} or Mg^{2+} in the protein molecule.

The B52 protease (Richardson, 1981) and the MC 60 protease (Barach *et al.*, 1978a) also had high Ca^{2+} ion content (8.1 and 4.2 g atoms per mole respectively) with lower values for Zn^{2+} , while neither Mg^{2+} nor Mn^{2+} were determined. In each case Zn^{2+} was believed to be essential for catalysis by maintaining the integrity of the active site, while Ca^{2+} appeared to stabilize the conformation of the molecule. The proteases of this study differed in that there was no apparent requirement for Zn^{2+} . If it is assumed that all metal ions have been removed from the protease by EDTA, then it is evident that the Ca^{2+} , Mg^{2+} and Mn^{2+} can play a dual role by maintaining the catalytic site activity and stabilizing the protein molecule.

4.5. Protease specificity

The comparison of the specificity of the proteases to a variety of protein substrates revealed that there was a marked preference for caseins (Table 3-18), although the activities were variable. For all proteases, except T16, α -casein was the better substrate. However, significant breakdown of the other caseins was also evident. The κ -casein was the most susceptible of the caseins to proteolysis by the T16 protease. There was no activity towards the whey proteins, α -lactalbumin

and β -lactoglobulin, by any of the proteases. Richardson and TeWhaiti (1978) studied the proteases of eight psychrotrophic bacteria and found that κ -casein was the most readily degraded κ -casein followed by γ -casein, β -casein and finally α -casein. Adams *et al* (1976) showed that, when their populations reached 10^4 cfu/ml, nine *Pseudomonas* species isolated from raw milk degraded κ -casein first, while β - and α -casein degradation was only detected at higher cell numbers ($10^6 - 10^7$ cfu/ml).

The inability of the proteases to break down the whey proteins is consistent with the findings for other psychrotroph proteases, which show that whey proteins are degraded slowly or not at all (De Beukelar *et al.*, 1977; Law *et al.*, 1977; and Richardson and TeWhaiti, 1978). This may be a result of the highly globular nature of whey proteins with their tight secondary and tertiary structures in comparison to the more random non-helical open structures of the caseins.

The proteases of this study were also capable of hydrolyzing non-milk proteins, such as bovine serum albumin, hemoglobin and ovalbumin (Table 3-18).

Five of the proteases were examined further to determine their ability to breakdown elastin and collagen. All exhibited collagenase activity and to a lesser extent, elastase activity (Table 3-19). Purified enzymes were used to eliminate the possibility that the breakdown of these substrates was the result of the activity of any minor proteases which may have been produced by the bacteria. There have been reports of extracellular proteases of *P. aeruginosa* strains which have elastolytic and collagenase activities, in addition to caseinase activity.

Such results indicate that these enzymes have a wide substrate specificity, with exceptionally good activity towards caseins. The presence of such enzymes in milk would be expected to cause proteolysis of the milk proteins resulting in deterioration and a shortened shelf-life of the product. Psychrotrophic proteases have been implicated in the development of bitter flavours, gelation, and decreased heat-stability of UHT sterilized milk (Cousin and Marth, 1977a; Richardson and Newstead, 1979; and Visser, 1981). In addition to the adverse effects on milk by direct proteolysis of the milk proteins by psychrotrophic proteases, these enzymes may play a role in the activation of plasminogen in milk to plasmin. Leytus *et al.*, (1981) demonstrated the conversion of plasminogen to its active form plasmin by a protease associated with the outer membrane of *E. coli*. Whether psychrotrophic proteases are capable of activating the zymogen of plasmin present in milk has yet to be determined. However the wide specificity of such proteases suggests that the possibility does exist.

The classification of microbial proteases into serine, thiol, metallo- and acid proteases can be further refined according to their side chain specificity (Moriyama, 1974). The metalloproteases may be classified into at least four groups: neutral and alkaline proteases and myxobacter AL-1 proteases I and II. The extracellular proteases of psychrotrophic pseudomonads may be either neutral proteases or alkaline proteases. The neutral proteases show specificity for hydrophobic or bulky amino acid residues, while the alkaline proteases have a very broad specificity.

The T16 protease is able to hydrolyze dipeptides which is unique for metalloprotease (Moriyama, 1974; Mitchell *et al.*, 1986). The inability of the T16 protease to hydrolyze z-tyrosyl-l-ser indicates that the amino acid residue at the carboxyl side of the splitting point can determine the primary proteolytic specificity, while the difference in specificity to z-tyrosyl-l-ala and z-tryptophyl-l-ala illustrates that the amino acid at the amino side of the splitting point also determines specificity (Table 3-20). This differs from the specificities of enzymes such as trypsin and chymotrypsin which are determined solely by the amino acid at the carboxyl side of the peptide bond which is cleaved.

Similar results were obtained using synthetic polypeptides by substituting the amino acid residues at either the N or C terminal (ala -- ile/leu; ala -- gly). The differences in specificities may be a result of both primary specificity, as well as secondary interactions, whereby amino acid residues more distant than those adjacent to the splitting point determine specificity.

Neutral metalloproteases have a requirement for hydrophobic or bulky amino acid residues at the amino side of the splitting bond. This would explain why the T16 protease was unable to hydrolyze z-tyrosyl-l-ser, because serine does not contain a hydrophobic side chain while alanine, threonine and leucine do (Bigelow, 1967). Based on these results the T16 protease can be classified as a neutral metalloprotease.

The T16 protease failed to hydrolyze TAME, a synthetic trypsin substrate, indicating that the enzyme does not possess esteryolytic activity like trypsin. The

protease was active against another trypsin substrate, BAPA, however the K_m (0.59 mM) was higher than that reported for trypsin. An assay method using BAPA would have limited value for detection of proteases in milk because of its low sensitivity. Such an assay may prove to be of some benefit, however, for enzyme kinetic studies because the reaction product, *p*-nitroanilide can be monitored as it is released during the reaction.

The pH-stat assay method also produces a readily detectable product of proteolysis (H^+) with the added advantage of being relatively sensitive when compared to the modified Hull's assay method (Fig 3-8). The limitations of the pH-stat method include its inability to accurately measure proteolysis at a pH of less than 8, as well as only being capable of assaying one sample at a time. In spite of these shortcomings the pH-stat method is presently the best available continuous assay method for the extracellular proteases of psychrotrophs from milk.

4.6. Effect of casein concentration on protease activity

The optimum concentration of soluble casein for the T16 protease activity was approximately 2.5 mg/ml, with slight inhibition at concentrations in excess of 5.0 mg/ml (Fig 3-6). The AFT 36 protease activity was linear in the range of 4 - 50 mg/ml, with a slight inhibition at 75 and 100 mg/ml (Stepaniak *et al.*, 1982). Alichandrais and Andrews (1977) reported that the AR11 protease was inhibited by sodium caseinate at concentrations greater than 5 mg/ml. They concluded that such proteases would be severely inhibited by the concentration of casein in

normal bovine milk (25 mg/ml). Although the T16 protease was inhibited to some extent at higher substrate concentrations, 77% of the maximum activity was retained at 25 mg/ml of soluble casein, and therefore would be expected to be quite active in milk. The inhibition which was noted at high casein concentrations may be a result of a decrease in available substrate because of an increase in the aggregate size of casein, or due to the accumulation of end-products.

4.7. Heat stability

Possibly the most outstanding feature of the T16 protease is its remarkable heat stability. Its ability to survive a temperature of 150°C for up to one minute illustrates why such proteases are of concern to the dairy industry. The D_{150} value (time to reduce activity by 90% at 150°C) of 2 minutes is comparable to other psychrotroph proteases. The protease of *Pseudomonas* sp. MC60 had a D_{149} value of 1.5 min (Adams *et al.*, 1975), while Mitchell *et al.* (1986) calculated that the D_{140} value of *Pseudomonas* OM41 was 5 minutes. The heat stability of these proteases are significantly greater than that of the native milk proteinase (plasmin) which has a reported D value of 7.10 s at 142°C (Alichandrais *et al.*, 1986).

The D values of 26.7 min at 90°C and 7.2 min at 120°C demonstrates that the T16 enzyme will survive typical pasteurization conditions (63°C/30 min or 71°C/15 s) as well as UHT processing (130-150°C/2-8 s).

4.8. Role of metal ions

Mayerhofer *et al* (1973) and Barach *et al.* (1976a) have reported that the heat stability of psychrotroph proteases is enhanced in milk. According to Barach and Adams (1977) the protective effect of milk could be eliminated by the addition of metal chelating agents or treatment with a cation exchange resin. Based on these results they concluded that divalent metal ions rather than substrate binding by milk proteins, as suggested by Mayerhofer *et al.* (1973), was responsible for the stabilizing effect of milk. Barach *et al.* (1976a) found that the MC60 protease required both Zn^{2+} and Ca^{2+} for optimal activity but only Ca^{2+} restored the protective effect of milk against high temperatures. Calcium was also effective in protecting the enzyme in buffer solutions lacking milk proteins.

The T16 protease was stabilized against inactivation by heat with the addition of exogenous Ca^{2+} or Mg^{2+} (Table 3-8). The role of the metal ions in heat stability may be to provide noncovalently bound ligands which stabilize the structure of the enzyme by linking ligands from different parts of the primary structure of the protein molecule. Preliminary circular dichroism studies have indicated that the conformation of the protease molecule becomes more random, or unordered, in the presence of the metal chelating agent, EDTA. A similar effect was reported for the thermophilic protease thermolysin (Feder *et al.*, 1971). Thermolysin lost its thermostability upon removal of calcium, however unlike the T16 protease, it still retained its catalytic properties.

Although calcium is known to increase the thermal stability of several

proteolytic, as well as other types of enzymes, most of these enzymes are readily inactivated at temperatures approaching 100°C (Ohta *et al.*, 1986). Therefore, the heat stability of psychrotroph proteases can not be attributed to metal binding alone.

4.9. Role of carbohydrate

Sugars are known to stabilize proteins against heat denaturation. Back *et al.* (1979) concluded that the stabilizing effects were a result of the hydrophobic interactions between the hydrophobic groups of the protein. No such protection to heat denaturation was afforded to the T16 protease by lactose as was noted with the added metal ions (Table 3-10). The relatively low average hydrophobicity of this protease (3322 J/mole) suggests that hydrophobic interactions may not play a significant role in maintaining the protein conformation in this case.

The T16 protease can be classified as a glycoprotein based on the presence of the amino sugars, glucosamine, and galactosamine, in the enzyme molecule. All six of the *Pseudomonas fluorescens* extracellular proteinases examined by Mitchell *et al.*, (1986) contained carbohydrate, however the types of sugars were not determined. The protease of *P. fluorescens* NCDO 2085 (AR11) lacked both galactosamine and glucosamine (Fairbairn and Law, 1986b) which indicates that not all such proteases can be considered to be glycoproteins.

The carbohydrate moiety of the protease may contribute to its heat stability. Hayashida and Yoshioka (1980) were able to demonstrate that the

removal of the covalently linked carbohydrate residues of two types of cellulases of a thermophilic fungus, *Humicola insolens*, resulted in a significant decrease in both thermal and pH stability. Whether the carbohydrate component of the psychrotroph proteases also acts as a stabilizer remains to be determined.

4.10. Protease renaturation

Other factors which may contribute to the heat stability of the T16 protease are those which contribute to the characteristic structure of the protein molecule. Like the thermostable protease of *B. thermoproteolyticus*, thermolysin, the T16 protease is stabilized by Ca^{2+} , lacks cysteine (i.e. has no disulphide linkages), and has a high content of low molecular weight amino acids such as glycine. These same properties are shared by other psychrotroph proteases (Barach and Adams, 1977; Richardson, 1981). The stabilization by Ca^{2+} rather than by disulphide bonds may allow the enzyme to become flexible upon denaturation by heat and then refold with the formation of Ca^{2+} salt bridges to its native conformation when the temperature is lowered. The small side chains of the low molecular weight amino acids would cause a minimum of steric hindrance upon refolding of the enzyme thus enhancing structural flexibility. Enzyme resistance to heat would therefore be based on its structural flexibility and capacity to accurately regain its native conformation, rather than having a rigid structure.

Such renaturation may explain the reactivation phenomenon which was noted for three of the proteases (Table 3-11) when stored on ice for 24 h after heating ($90^\circ\text{C}/10 \text{ min}$). A number of proteins are known to renature upon

storage following heat treatment (Reichert and Fung, 1976; Carel and Baldwin, 1973). Reactivation of the phosphatase enzyme in milk, following pasteurization, has been reported (Richardson *et al.*, 1964). Edmondson *et al.* (1966) found that phosphatase reactivation in sterilized whole milk amounted to as much as 50% of the raw milk activity after 2 to 4 days of storage at room temperature. The phosphatase enzyme required both Ca^{2+} and Mg^{2+} for optimum reactivation while the addition of EDTA inhibited reactivation. This led Richardson *et al.* (1964) to propose that the heating process resulted in Ca^{2+} and Mg^{2+} being forced into colloidal form resulting in the separation from the enzyme. This dissociation may have caused a temporary increase in the heat stability of the apoenzyme. Upon storage at lower temperatures sufficient calcium and magnesium returned to the ionic state and recombined with the apoenzyme to form an active enzyme.

The reactivation of the T16 protease does not appear to be a result of an increased availability of metal ions due to a shift from colloidal to ionic forms upon cooling. The addition of exogenous Mg^{2+} and Ca^{2+} soon after heating was unable to reactivate the enzyme following a one hour preincubation period at 5°C. It would appear that the storage time required to allow the enzymes to renature is a critical factor for reactivation. The effect of storage temperature on the time required and degree of renaturation was not determined.

Should the renaturation phenomenon be a common feature of other psychrotroph proteases, the practice of assaying milk for protease activity soon

after pasteurization would significantly underestimate the spoilage potential of the heat stable proteases present.

4.11. Effect of temperature on secondary structure of T16 protease

It is generally accepted that the circular dichroism (CD) spectrum of a protein is a direct reflection of its secondary structure (Hennessey and Johnson, 1981). The CD spectrum of the purified T16 protease at both 5 and 25°C (pH 7.2) were identical and could be considered to represent the native conformation of the enzyme. The protein was primarily "random" coil with approximately a third of the molecule consisting of β -structure while little or no α -helix was detected. There have been no other reports of the secondary structure analysis of psychrotroph proteases, however the conformation of the thermophilic enzyme thermolysin has been elucidated and showed some similarities to T16 protease. Thermolysin is a globular protein consisting of β -structures and non-periodic regions with a single small region of α -helix (Matthews *et al.*, 1974). The authors noted however that aside from the four Ca^{2+} bound to the protein, there was nothing unique about thermolysin's conformation which would account for its heat stability.

Denaturation of the T16 protease was apparent at temperatures in excess of 35°C as indicated by the unfolding of the protein molecule (Fig. 3-17) with maximum unfolding at 50-55°C. This corresponded to the temperature at which the T16 protease activity was inactivated to a greater extent than at either 90 or

120°C (Fig. 3-10) and is known as low temperature inactivation (LTI). West *et al.* (1978) proposed that LTI of heat-stable psychrotroph proteases is the result of a conformational transition of the protease which opens up the enzyme structure at 55°C. This is supported by the CD spectra of protease T16 at temperatures between 45 and 55°C, which indicates that the protease structure tends to become more random. The conformational change is believed to cause a reversible loss of catalytic activity and susceptibility to proteolysis by other protease molecules yet to be denatured (autolysis). The protease may also form aggregates with the casein micelles of milk, presumably by the exposure of internal nonpolar side chains on the protease to the highly hydrophobic casein proteins. Such hydrophobic interactions would not be favoured at temperatures greater than 60°C (Barach *et al.*, 1978). The inhibition of protease T16 activity at 50°C can not be a result of adsorption of protease to casein micelles, since no milk proteins were present during the heat treatment. The inactivation was probably a result of autodigestion. At temperatures higher than 55°C the T16 protease appears to undergo an additional conformational change, to a more ordered structure. This is contrary to the view of Stepaniak and Fox (1983) who suggested that at higher temperatures the protease molecule unfolds to a greater extent than at 55°C, which renders the enzyme proteolytically inactive. The protease at higher temperatures would therefore not be susceptible to autolysis.

It is unlikely that the T16 is refolding to its original conformation with increased temperatures. The increased folding may result from a general rearranging of ionic groups to form a more ordered protein molecule by ionic binding, however, it may be catalytically inactive.

Further studies using other techniques (optical rotary dispersion, X-ray crystallography) for the determination of protein secondary structure are needed to confirm these results obtained by circular dichroism.

4.12. Antigenic relatedness

All six of the proteases produced precipitin bands in the Ouchterlony double-diffusion tests against anti-T16 protease IgG. In each case the arcs converged without spur formation indicating that these enzymes are antigenically similar and may be structurally homologous. However, the antigenic portions of each protease may only represent a small fraction of the entire molecule. Therefore, caution must be exercised when estimating structural similarity based on antigenic relatedness.

The anti-T16 protease IgG was an effective inhibitor of each of the proteases, although the amount of antibody required to inhibit protease activity was greater than expected for such a specific reagent. This may mean that the immunoinhibition was not necessarily a result of specific binding of antibody to the active site but probably involves interactions with other sites on the molecule. The non-competitive nature of the inhibition of the T16 protease by the IgG fraction indicates that the antibody was not competing with the binding of substrate to the catalytic site. The loss of activity may be a result of immunoprecipitation of the protease by the antibody, which would effectively remove the enzyme from its substrate by taking it out of solution.

The proteases of psychrotrophic pseudomonads from Ontario, British

Columbia, Ireland, U.S.A., Australia, and Netherlands, also cross-reacted with the antiserum to the T16 protease. Six of the crude extracts produced multiple bands (015, 240, S63, M5, BC 1-1, BC 2-2) which may suggest that more than one protease is produced. Although the majority of *Pseudomonas* species produce only one extracellular protease, there have been reports of those which produce a number of proteases (Peterson and Gunderson, 1960; Wretling and Wadstrom, 1977; Jensen *et al.*, 1980; and Alichandrais and Andrews, 1977). *P. fluorescens* AFT 36 and *Pseudomonas* AFT 21 are both able to produce three distinct proteases, although the proportion of each depended on the growth conditions (Stepaniak *et al.*, 1982; and Stepaniak and Fox, 1985). Interestingly, only a single precipitin arc was noted (Fig 3-20) for each of the AFT 21 and AFT 36 extracts in this study. It may be that conditions were not appropriate for the production of more than one protease, or that the other proteases did not react with the antiserum. Another possibility is that the resolution of the precipitin arcs was not sufficient to facilitate their observation.

The broad specificity of the antibodies to the T16 protease for other psychrotroph proteases indicated that they may be useful in the development of a sensitive immunoassay for the detection of such enzymes in milk. The enzyme-linked immunosorbent assay (ELISA) technique using horseradish peroxidase conjugated to the anti-T16 protease IgG was of limited value. Although it was possible to detect the presence of the T16 protease, the sensitivity of the immunoassay was much less than that for modified Hull's assay method, 720 $\mu\text{g/ml}$ (240 $\mu\text{g/assay}$) and 500 $\mu\text{g/ml}$ (50 $\mu\text{g/assay}$) respectively. A low conjugation

efficiency between the peroxidase enzyme and the IgG may account for the relatively low sensitivity. Birkeland *et al.* (1985) were able to detect protease concentrations as low as 0.25 ng/ml in raw milk using an ELISA method with an alkaline phosphatase conjugate. However, they noted that some psychrotrophic strains produce immunologically unrelated proteases which could not be detected, illustrating the limitation of the immunoassay.

4.13. Protease localization

The production of extracellular proteases requires the transport from an intracellular site of synthesis to an exterior location and release in an active form. The active protease of *P. fluorescens* T16 was only detected in the culture medium. No protease activity was associated with the intracellular region of the cell, i.e. the cytoplasm, which indicates that this heat-stable protease is an extracellular enzyme. Most enzymes secreted by Gram-negative bacteria are found in the periplasmic space (zone between the cytoplasmic membrane and the cell wall) or in the outer cell wall membrane (Ramaley, 1979). Osmotic shock treatment or spheroplast formation releases such enzymes into the medium without the release of cytoplasmic enzymes.

When the washed cells of the T16 isolate were subjected to shock treatment no protease activity was detected in either the periplasmic or cytoplasmic fractions. However, the contents of the periplasmic fraction did cross-react with the T16 antiserum to form a precipitin band. This may indicate the presence of an inactive form (proprotease) of the protease. These findings are consistent with

those found by Jensen *et al.* (1980) for the extracellular protease of a clinical isolate of *P. aeruginosa*. They were able to demonstrate the presence of an inactive, periplasmic-associated precursor to the extracellular protease. The precursor was antigenically related to the major extracellular protease and could be converted to an active form by limited proteolysis with the active protease, as well as trypsin.

Unlike the precursor isolated from *P. aeruginosa* it was not possible to activate the "precursor" of the T16 protease, with either active protease or trypsin. The possibility exists that the concentration of precursor may have been so low at the time that the cells were harvested (3 days) that any conversion of proprotease to protease was relatively small and difficult to detect. Jensen *et al.* (1980) found that the precursor material was highest in cells grown up to 10 to 12 hours, followed by a rapid decline after 15 hours.

It has been suggested that extracellular proteases are synthesized in precursor form to facilitate passage through the cytoplasmic membrane. This is in agreement with the "signal hypothesis" of protein secretion (Emr *et al.*, 1980) which states that a protein destined to be secreted is initially synthesized as a larger precursor with 15-30 additional amino acids at the amino terminal end of the molecule on membrane-bound ribosomes. The polypeptide chains, led by the signal sequence, are transferred directly through the membrane to their extracellular location. In most cases secreted enzymes of Gram-negative bacteria are activated by limited proteolysis by proteases in the cell membrane or in the periplasmic space (Ramaley, 1979).

The extracellular protease of *P. aeruginosa* appears to have an intermediate stage in which the precursor remains in the periplasm until it is released into the culture medium and converted to active protease. This mechanism may also be a characteristic of other pseudomonads such as isolate T16, although the actual method by which the precursor is activated may differ.

4.14. Mole percent G+C

A comparison of the mean base composition of DNA for the psychrotroph isolates from different geographical regions was expressed as mole percent G + C (%G+C). Only differences in %G+C are significant and they indicate genomic difference (Jones and Sneath, 1970). Similarity in %G+C does not necessarily indicate genomic similarity of the 20 isolates examined. Only O15 was found to differ significantly (by 14-18%) from the rest with a %G+C value of 44. According to DeLey (1973) a difference of 20 to 30% in G+C ratio means that there are practically no nucleotide sequences in common between two organisms. On the basis of this criterion the O15 isolate probably belongs to a different genus from the others, possibly *Alteromonas*. The values obtained for the other isolates are well within the range for members of the genus *Pseudomonas* (58-70 %G+C).

Chapter 5

CONCLUSIONS

This study has shown that affinity chromatography using CBZ-D-phenylalanine TETA Sepharose 4B offers a fast and efficient technique for the purification of the six psychrotrophic pseudomonad heat-stable proteases. These proteases are neutral metalloproteases with a requirement for divalent cations for both stability and catalytic activity. They are quite heat resistant and would be expected to survive HTST pasteurization and even UHT sterilization temperatures. The heat resistance appears to be based on the flexibility and renaturation of the protease molecule, rather than maintenance of a rigid structure.

The conditions in pasteurized milk are favourable for those proteases which remain active after heat treatment. The proteases exhibit a high affinity for caseins (particularly alpha- and kappa-caseins) as a protein substrate, and the pH of milk is near the optimum for protease activity. Considerable activity is retained at room temperature (25°C), the temperature at which UHT milk is usually stored. Significant levels of protease activity are also evident at refrigeration temperatures. This combination of heat stability and retention of proteolytic activity at low incubation temperatures, indicates that such proteases may play a major role in the reduction of the shelf-life of milk and milk products.

Overall, the proteases possess mutually similar physicochemical properties and appear to be antigenically related.

From the standpoint of its heat resistance there is nothing unique about the T16 protease's conformation. The protease consists of a single polypeptide which has a secondary structure consisting primarily of "random coil". Such a conformation would impart some degree of flexibility to the molecule and further supports the hypothesis that the heat-stability is based on the flexibility and renaturation of the protease molecule, rather than maintenance of a rigid structure.

The active T16 protease is exclusively extracellular, although an inactive form of the enzyme may exist in the periplasmic region of the bacterial cell. The mechanism by which an inactive form of the protease could be converted to its active form has yet to be determined.

Further research is required into a number of different areas dealing with heat-stable extracellular proteases. This includes elucidation of the factors which affect protease production by psychrotrophs, as well as an understanding of protease production at the molecular level. Technologies need to be developed for use by the dairy industry to inhibit the growth and/or protease production of psychrotrophs during refrigerated storage of milk and milk products. The use of controlled or modified atmosphere storage, or biological control with psychrotroph-specific bacteriophages have been used to inhibit psychrotrophs in meat, and may be applicable to milk and milk products.

A sensitive protease assay method is required which can be used by those in the dairy plant to quickly and accurately determine the protease content of raw and processed milk. Such an assay method would aid in assessing the quality and potential shelf-life of dairy products.

An aspect which has received little attention to date is the potential industrial applications of these proteases. Proteolytic enzymes are becoming increasingly important to the food, chemical and pharmaceutical industries. The unique properties of the heat-stable proteases may allow these enzymes to meet an existing or future need by such industries.

Chapter 6

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Chapter 7

APPENDICES

7.1. Appendix A: Preparation of hemoglobin-sepharose affinity chromatography material

The Sepharose 4-B suspension (125 ml) was mixed with an equal volume of deionized water, followed by 12.5 g of cyanogen bromide dissolved in 125 ml of deionized water. The pH was immediately adjusted to 11 by titration with 4 N sodium hydroxide. After 10 min the activated sepharose was washed with 20 volumes of cold 0.1 N sodium bicarbonate solution on a Buchner funnel using mild suction. The washed Sepharose was suspended of 0.1 N sodium bicarbonate (pH 9.0) and 50 ml of 4% hemoglobin solution was quickly added. This mixture was stirred gently at 2-3°C for 24 h and washed extensively with deionized water.

7.2. Appendix B: Preparation of reagents for Lowry's method of protein determination

Reagents:

- A. 2% sodium potassium tartarate in deionized water
- B. 1% copper sulphate in deionized water
- C. 0.2 N sodium hydroxide in deionized water
- D. 4% sodium carbonate in deionized water
- E. 1 N Folin-Ciocalteu Reagent

The reagents were mixed as follows:

Solution I : 25 ml of Reagent C and 25 ml of Reagent D

Solution II : 1 ml of Reagent A and 1 ml of Reagent B

Solution III : Add 1 ml of Solution II to 50 ml of Solution I

Solution IV : Add 1.0 ml of deionized water to 1.0 ml of Reagent E

7.3. Appendix C: Ornstein-Davis polyacrylamide gels

Solutions :

A. Acrylamide 30 g, bisacrylamide 0.8 g made up to 100 ml with deionized water

B. Tris base 18.2 g, 6 N HCl 20 ml made up to 100 ml (pH 8.8)

C. Tris base 6.1 g in 50 ml of water adjusted to pH 6.8 with 6 N HCl and then made up to 100 ml.

D. Tris base 3 g, glycine 14.4 g made up to 100 ml, pH 8.3 (reservoir buffer).

E. Glycerol 10 ml, 2-mercaptoethanol 5 ml, 12.5 ml of solution C made up to 100 ml with deionized water (sample buffer).

F. Ammonium persulphate 0.2 g in 2 ml of deionized water

7.5% gels were prepared as follows :

Lower Gel

water 19.7 ml

soln B 10.0 ml

soln A 10.0 ml

soln F 0.25 ml

TEMED 15.0 μ l

Upper Gel

water 3.25 ml

soln C 1.25 ml

soln A 0.50 ml

soln F 1.0 μ l

TEMED 5 μ l

(TEMED = N,N,N,N-tetramethylethylenediamine)

7.4. Appendix D: Weber and Osborn SDS polyacrylamide gels

Reagents:

1. Gel Buffer : 7.8 g NaH_2PO_4 , 33.6 g Na_2HPO_4 , 2.0 g SDS, water to make 1000 ml (pH 7.0).

2. Acrylamide Solution : 22.2 g acrylamide, 0.6 g methylene bisacrylamide water to make 100 ml.

The solution was filtered through Whatman No.1 filter paper and stored in a dark bottle at 4°C.

3. Ammonium Persulphate Solution : 30 mg ammonium persulphate in 2 ml of water.

4. Dialysis Buffer: 10 mM sodium phosphate buffer, 1 g SDS, and 1 ml 2-mercaptoethanol.

To prepare 10% gels:

Reagent 2 13.5 ml

Reagent 1 15.5 ml

Reagent 3 1.0 ml

TEMED 45 μl

7.5. Appendix E: Preparation of low phosphate medium

Low phosphate growth medium was prepared as described by Jensen *et al.*, (1980).

0.5% neopeptone (Difco)

0.25% yeast extract (Difco)

0.1% glucose

1mM CaCl_2

adjusted to pH 7.2 with 0.1 N HCl

7.6. Appendix F: DNA purification

DNA purification was performed by a modification of the method of Marmur (1961).

Reagents:

- (1) saline-EDTA (150 mM sodium chloride, 100 mM EDTA in deionized water, pH 8.0)
- (2) lysozyme (20 mg/ml in deionized water)
- (3) 25% sodium dodecyl sulphate (SDS) in deionized water.
- (4) 5 M sodium perchlorate in deionized water
- (5) chloroform: isoamyl alcohol (24:1)
- (6) absolute ethanol
- (7) dilute saline citrate (15 mM sodium chloride, 1.5 mM sodium citrate, in deionized water, pH 7.0)
- (8) concentrated saline citrate (1.5 M sodium chloride, 150 mM sodium citrate in deionized water, pH 7.0)
- (9) ribonuclease (1 mg/mL in 150 mM sodium chloride in deionized water; boiled for 10 min)

Procedure:

- (1) A 100 ml culture was grown to the late log phase in TSB at 25°C
- (2) The cells were pelleted by centrifugation (7,000 x g for 10 min) and suspended in 5 ml of saline-EDTA by stirring vigorously.
- (3) The cells were disrupted by adding 0.5 ml of lysozyme and shaken in a 37°C waterbath until the solution became clear and viscous.
- (4) 0.4 ml of 25% SDS was added, shaken gently to mix, and then placed in a waterbath at 60°C for 5 min. Then cooled to room temperature.
- (5) 1.4 ml of 5 M sodium perchlorate was added, followed by 7 ml of chloroform: iso-amyl alcohol. The tube was capped and shaken vigorously for 10 min.
- (6) Centrifuged at 10,000 x g for 15 min. Two layers were formed.
- (7) Removed the upper aqueous layer and placed it in a sterile tube. The interface material and the lower layer were discarded.
- (8) 15 ml of ice cold ethanol was gently layered on top of the aqueous layer. A glass rod was inserted to the bottom of the tube and rotated quickly to wind the DNA fibers around the rod.
- (9) The DNA fibers were removed and dissolved completely in 4.5 ml of dilute saline citrate solution.

(10) 0.25 ml of DNase-free ribonuclease was added, shaken gently and incubated at 37°C for 15 min. Then 0.5 ml of concentrated saline citrate solution was added.

(11) Shaken for 5 min with an equal volume of chloroform: isoamyl alcohol, centrifuge at 10,000 x g for 15 min and remove the aqueous layer was removed as before.

(12) 15 ml of cold ethanol was added to the upper layer and allowed to stand for 5 min on ice.

(13) The resulting purified DNA was picked up with a sterile wire-loop and dissolved completely in 4.5 ml of the dilute saline citrate solution.

7.7. Appendix G: Purification of immunoglobulin G (IgG)

The IgG fraction of the antiserum to the anti-T16 protease was prepared according to the methods of Kekwick (1940) and, Levy and Sober (1960).

Reagents:

- (a) 25% (w/v) sodium sulphate
- (b) 0.85% sodium chloride (saline)
- (c) sodium phosphate buffer 17.5 mM, pH 6.3

Procedures:

(1) Precipitation with 18% Sodium Sulphate

To 20 ml of whole serum was added 51.4 ml of 25% sodium sulphate with constant stirring at room temperature. The mixture was centrifuged at 10,000 x g for 10 min at room temperature. The pellet was dissolved in a measured amount of saline. The volume of the pellet was determined by subtracting the volume of saline added from the final volume obtained.

(2) Precipitate with 16% Sodium Sulphate

The amount of 25% sodium sulphate added was determined by;

$$\text{volume sodium sulphate} = 1.78 (\text{final volume}) - 2(\text{pellet volume})$$

The sodium sulphate was added with constant stirring. The mixture was centrifuged at $10,000 \times g$ for 10 min 25°C . The pellet was dissolved in saline and dialyzed overnight against 17.5 mM sodium phosphate buffer pH 6.3 at 5°C .

(3) DEAE Cellulose Chromatography

The dialyzed protein was applied to a DEAE cellulose column (10 mg protein/ ml column material) which had been equilibrated to pH 6.3 with 0.2 M sodium dihydrogen phosphate and washed several times with 17.5 mM sodium phosphate buffer pH 6.3 at 25°C . The absorbance in each of the fractions was monitored at 280 nm. The peak fractions which contained the gamma immunoglobulin (IgG), were pooled and the protein content determined.

7.8. Appendix H: Preparation of Ouchterlony double-diffusion and immunoelectrophoresis slides

The slides for both the Ouchterlony double-diffusion, and immunoelectrophoresis tests were prepared the same way.

Adhesive Agar:

0.1 g Noble agar, 0.05 g glycine, 100 ml deionized water (boil to dissolve)

Running agar:

1.5 g Noble agar, 75 ml deionized water 25 ml of 60 mM tris-barbitol buffer 0.02% sodium azide (boil to dissolve)

Procedure:

Clean glass slides (25 x 75 mm) were coated with a thin layer of melted adhesive agar and allowed to solidify in a 37°C incubator. The slides were then overlaid with the running agar and again incubated at 37°C to allow the agar to solidify.

Tris-barbitol buffer was prepared by dissolving 1.5 g of tris-barbitol, pH 8.8 (High Resolution Buffer; Gelman Sciences, Inc.) in 98.2 ml of deionized water.

7.9. Appendix I: Conjugation of horseradish peroxidase to anti-T16 IgG

The following method of Nakane and Kawaoi (1974) was used to prepare the horseradish peroxidase anti-T16 protease IgG conjugate.

1. 5 mg HRPO (Sigma Type VI, RZ 3.0) was dissolved in 1.0 ml of 300 mM sodium bicarbonate, pH 8.1.

2. Addition of 0.1 ml of 1% fluorodinitrobenzene in absolute alcohol.

3. Mixed gently for 1 h at room temperature, followed by the addition of 1.0 ml of sodium periodate in deionized water.

4. Mixed gently for 30 min at room temperature, followed by the addition of 1.0 ml of 160 mM ethylene glycol in deionized water.

5. Mixed gently for 1 h at room temperature, and dialyzed against three 1-litre changes of 10 mM sodium bicarbonate pH 9.5, at 4°C.

6. 5 mg of anti-T16 IgG in 1.0 ml of 10 mM sodium bicarbonate, pH 9.5 was added to 3 ml of the HRPO-aldehyde solution.

7. Mixed gently for 2-3 h at room temperature to which 5 mg sodium borohydride was added.

8. Dialyzed at 4°C against phosphate-buffered saline (PBS).

9. Precipitate which may have formed was removed by centrifugation at 7,000 x g for 10 min.

10. The solution was applied to a Sephadex G-100 (85 x 1.5 cm) column, equilibrated in PBS.

11. The absorbance was read in each of the 3 ml fractions at 280 and 403 nm.

12. The first peak eluted, contained the HRPO-labelled IgG.

13. The HRPO conjugate IgG was stored in the presence of 2 mg BSA/ml at -20°C.

